The ecological interactions of the hemiparasite *Rhinanthus minor* and its invertebrate herbivores

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Thesis submitted for the degree of

Doctor of Philosophy

University of Sussex

July 2010

University of Sussex

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Summary

The hemiparasite *Rhinanthus minor* is a common component of many northern temperate grasslands. It can have major impacts on ecosystem processes, and is often present at very high densities, therefore constituting an important potential food source for invertebrate herbivores. Thus, the aim of this thesis is to investigate the interaction between this hemiparasite and its invertebrate herbivores, and to explore the various ecological factors which are likely to affect this interaction.

In the first series of experiments the thesis explores how the density of the hemiparasite affects the composition of the vegetation, the performance of the hemiparasite and the levels of invertebrate herbivore damage it receives. The results of a field experiment and a greenhouse study demonstrated that hemiparasite density can adversely affect its own performance and survivorship and dramatically change the composition of the vegetation, but surprisingly appeared to have no impacts on the levels of herbivore damage the hemiparasite receives.

The second series of experiments investigated the impacts if host identity on the performance of the hemiparasite and how this affects its invertebrate herbivores. The results demonstrated that host identity can have a major impact on the performance of the hemiparasite and its herbivores; however, the indirect effects on the invertebrates appear to be species specific.

Thirdly, the thesis examines the effects of multiple host plants on the performance of *R. minor* and the knock-on effects for its invertebrate herbivores. Experiments demonstrated that multiple hosts are beneficial to *R. minor*, and that the anti-herbivore defensive properties conferred to the hemiparasite by certain host plants are maintained in the presence of a second host species.

Finally, the impact of nutrient addition and host plant damage on the performance of the hemiparasite and on the performance of its invertebrate herbivores was examined. The experiments showed that while certain host plants have highly contrasting effects on the performance of the hemiparasite's herbivores, the addition of nutrients and impact of host plant damage largely remove these differences, while neither factor appeared to affect the performance of the hemiparasite.

Declaration

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another University for the award of any other degree.

All of the experiments, analysis and interpretation described within this thesis are my own work.

Signature:..... Date:.....

Acknowledgments

Firstly I would like to thank my supervisors Sue Hartley and Alan Stewart, their help and guidance during this DPhil has been invaluable. I am grateful to the Natural Environment Research Council for funding this project.

I would like to thank all my friends and colleagues at the University of Sussex for their encouragement and advice over the last few years. These include Sam, John, Marina, Steph, Jan, Naomi, Lynne, Eric, Jonathan, Rosie, Claudia, all the other member of the ecology group and many other friend within the department that have made it such a pleasurable place to work. I would especially like to thank Sam, both for his friendship and for all his practical help. I would like to thank Dianne Ellis for all her help, especially with the C:N analyser.

I would also like to thank my family, for their encouragement, interest and patience in my endeavours, and I would like to thank my parents for their belief in my abilities. My two children, Emily and Joshua, who were both born while I have been working towards this DPhil, provide me with all the inspiration I should ever need. Finally and most importantly, I would like to thank my wife Esther for her support, especially during the difficult times, without her I would not have come so far.

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Chapter 1. General Introduction

1.1 An Introduction to Parasitic Plants

Parasitic organisms, or organisms that are partly or wholly dependent on other organisms for their nutritional needs while supplying no obvious benefit to their hosts, have long been recognised as important components in ecological communities. Parasitic plants are no exception and have been shown to have major impacts not only on their host plants but on their host plant communities, on invertebrate herbivores and on numerous other aspects of their community (Press and Phoenix, 2005, Irving and Cameron, 2009). Furthermore parasitic angiosperms are extremely diverse and account for approximately one percent of all known plants species (in excess of 3000 species) (Atsatt, 1983, Nickrent *et al.*, 1998). They are believed to have evolved independently 11 times from three different subclasses, and are distributed across 22 families (Nickrent *et al.*, 1998).

This introduction is intended to provide a brief overview of the ecology of parasitic plants and their importance in community structure before focussing more specifically on the species which is the central subject of this thesis, *Rhinanthus minor*.

1.1.1 Host-Parasite Interactions

Despite their considerable diversity and wide range of evolutionary pathways, all parasitic plants share a common mode of host attachment; haustoria (Kuijt, 1969, Riopel and Timko, 1995). This organ allows parasitic plants to form attachment to the vascular system of their host by means of a specialised endophyte which forms a

connection to the host plants vascular tissue (Riopel and Timko, 1995, Kuijt, 1969). Once established, a parasitic plant can remove either phloem or xylem sap by maintenance of a superior net sink strength (Press *et al.*, 1999, Stewart and Press, 1990, Jiang *et al.*, 2003).

The haustorial attachments therefore, allow parasitic plants to remove large amounts of water (Ehleringer and Marshall, 1995, Jiang *et al.*, 2003) together with numerous minerals (Pate, 1995, Jiang *et al.*, 2004b) and organic solutes from their hosts (Jiang *et al.*, 2004a, Jiang *et al.*, 2005a, Jiang *et al.*, 2005b). Unsurprisingly, this can have major impacts on the physiology and biochemistry of the host plant (Stewart and Press, 1990), resulting in a decreased rate of photosynthesis (Cameron *et al.*, 2008), and often a significant reduction in the hosts biomass and/or performance (Matthies, 1995, Seel and Press, 1996, Tennakoon and Pate, 1996). Indeed, these impacts can be so severe that the parasitic weeds of the genera *Striga* and *Orobanche* (commonly known as witchweeds and broomrapes) are considered to be serious pests of several important food crops and are of considerable economic and humanitarian importance (Musselman, 1980).

Parasitic plants can be divided into number of categories based on the interactions with their hosts. The first division can made on the basis of their point of attachment; species that form parasitic attachments with the roots compared to those that form parasitic attachments with the above-ground portion of the host. Secondly, parasitic plants can be divided into those that are capable of autotrophic photosynthesis, called hemiparasites, and those that are completely lacking photosynthetic apparatus, called holoparasites. While, all holoparasites are completely reliant on their host because they lack ability to photosynthesise, not all hemiparasites require a host to complete

their life cycle. Therefore the hemiparasites must be further subdivided into those that are completely dependent on their host plant, termed obligate hemiparasites and those that are not, termed facultative hemiparasites (Press, 1989).

Very few parasitic plants appear to be exclusively associated with a single host plant species (but see Musselman and Press, 1995, Kuijt, 1969), and the majority appear to be able to attach to wide range of plants, often from several different families (Kuijt, 1969, Nilsson and Svensson, 1997, Gibson and Watkinson, 1989). Despite this ability it appears that not all host plants are equally affected by their parasites (Cameron *et al.*, 2008). Furthermore, there is considerable variation in the performance of parasitic plants associated with different host species (Seel *et al.*, 1993a, Matthies, 1996). One of the principal reasons for this variability appears to be related to the ability of some hosts plants to defend themselves physically from the developing haustoria of the parasite. This is achieved either by localised cell death or via the production of a lignified barrier around the developing haustoria (Cameron *et al.*, 2006, Arnaud *et al.*, 1999, Labrousse *et al.*, 2001, Rumer *et al.*, 2007).

In addition to the physical defences produced by some host plants, the performance of a parasitic plant is likely to be affected by the types and quantities of organic and inorganic solutes removed from its host plants. For example, Govier *et al.* (1967) demonstrated a large variation in the range of amino acids and other organic molecules obtained from two host species of the hemiparasite *Odontites verna.* Further variation in the performance of parasitic plants associated with various host species is undoubtedly related to the particularly poor ability of many parasitic plants to assimilate inorganic nitrogen (Seel *et al.*, 1993b). Therefore, parasitic plants associated with nitrogen-rich hosts, for example legumes, may be expected to have

an enhanced performance when compared to parasitic plants associated with nitrogen poor hosts such as grasses (Seel *et al.*, 1993a).

1.1.2 The Impacts of Parasitic Plants on Plant Communities

Parasitic plants are believed to be present in almost all terrestrial ecosystems (Musselman and Press, 1995) suggesting their impacts on ecosystems processes are of considerable importance. In addition to their abundance and diversity, the variable and species-specific interactions between parasitic plants and their host plants mean that parasitic plants can have major impacts on plant communities. The most consistent impact of parasitic plants on plant communities appears to be a reduction in overall host plant biomass (Ameloot *et al.*, 2006, Pennings and Callaway, 2002, Pywell *et al.*, 2004, Davies *et al.*, 1997). For example, a meta-analysis by Ameloot *et al* (2005) demonstrated that hemiparasites in the genus *Rhinanthus* reduced the above ground biomass of the host plant community by between 40 and 60 %, on average. When taking the biomass of the hemiparasite itself into account, overall above ground biomass was found to be reduced by 26 % on average. In addition to this, the presence of parasitic plants can benefit some plant species over others (Suetsugu *et al.*, 2008) and tends to lead to an overall increase in plant diversity (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Davies *et al.*, 1997). The principal reason for these increases appears to be due to a reduction in the dominant plants in the system, thus allowing the subordinates to gain an advantage (Niemela *et al.*, 2008). Furthermore, gaps in the vegetation left by annual hemiparasites may provide micro-sites for seedlings to establish (Pywell *et al.*, 2004). Conversely, Gibson and Watkinson (1992) found that the presence of the hemiparasite *Rhinanthus minor*

could actually decrease plant diversity in certain systems because the hemiparasite was selectively parasitizing the competitively inferior species. Whether their presence leads to an increase or decrease in diversity it is clear that, in many cases, the presence of parasitic plants can have large impact on the composition of the plant community.

1.1.3 Interactions Between Parasitic Plants and Invertebrate Herbivores

The growth rate and reproductive output of invertebrate herbivores are known to be affected by a multitude of factors (Hartley and Jones, 1997). In particular, the amount of plant material available, its nutritional quality and the concentration of nitrogen are all believed to be important factors determining the performance of invertebrate herbivores (Price, 1991, Mattson, 1980, White, 1984). Parasitic plants have a significant impacts on all of these factors, causing major reductions in host plant biomass (Ameloot *et al.*, 2005) and removing large quantities of water and solutes from their host plants (Jiang *et al.*, 2003, Jiang *et al.*, 2004b). In addition, the attachment of parasitic plants leads to an overall reduction in the amount of amino acids in the vascular systems of the host and causes changes to the ratios of certain amino acids (Seel and Jeschke, 1999). This may also have large impacts on the performance of invertebrate herbivores. Herbivores are also known to be greatly affected by the presence of a wide range of chemical defences (Bennett and Wallsgrove, 1994). Again, it would seem likely that hemiparasites may influence the concentrations and types of plant chemical defences because of their impacts on the host plant's nutrient status.

It is perhaps unsurprising, therefore, that the presence of parasitic plants has been shown to affect herbivores feeding on a shared host indirectly (Bass, 2004, Puustinen and Mutikainen, 2001). Bass (2004) demonstrated that the presence of a hemiparasite significantly increased the mortality of an aphid feeding on the grass species *Poa annua*, while Puustinen and Mutikainen (2001) suggest that the presence of a hemiparasite may decrease levels of secondary metabolites in the host plant leading to increased herbivore damage. Furthermore, given the large impacts that parasitic plants can have on plant communities it would seem likely that their presence could modify entire insect communities.

While it would seem likely that the parasitic plants can have major impacts on the interactions between host plant and their invertebrate herbivores, the interaction between invertebrate herbivores feeding on parasitic plants and the identity of the host also warrants further consideration. In many cases parasitic plants may represent important, and nutrient rich, food sources for herbivores (Watson, 2001, Ehleringer *et al.*, 1986). Firstly, it would seem likely that the amount of nutrients, and in particular the amount of nitrogen, acquired by the parasite from its host plant would impact on the performance of its invertebrate herbivores (Mattson, 1980, White, 1984). In addition to this, some host plants transfer defensive secondary metabolites to their parasitic plants (Wink and Witte, 1993, Rasmussen *et al.*, 2006, Stermitz *et al.*, 1989, Adler and Wink, 2001, Adler, 2000), which can significantly reduce the performance of the parasitic plant's invertebrate herbivores (Marvier, 1998, Marvier, 1996). Furthermore, it seems that this transfer of secondary metabolites is hard to predict, for example Schadler *et al.* (2005) demonstrated that the host's toxicity is not a good indicator of whether a host plant will transfer toxicity to its plant parasite. Therefore, although parasitic plants potentially provide an alternative food source for

invertebrate herbivores, their palatability and nutritional quality may vary enormously depending upon which host plant species they are attached to.

1.2 *Rhinanthus minor*

1.2.1 Taxonomy and Distribution

The hemiparasite *R. minor* is an extremely common component of many grassland ecosystems, and is most commonly associated with meadows and pastures (Grime *et al.*, 1988). *R. minor* is distributed across a large part of the temperate northern hemisphere including most of northern Europe as far north as Iceland, a large portion of North America and parts of Asia. It is however, absent from many Mediterranean regions, presumably due to the mild winters and relatively dry conditions (Westbury, 2004).

The genus *Rhinanthus* was considered, up until recently, to form a part of the Scrophulariaceae or figwort family (Stace, 1997, Kuijt, 1969). Recent molecular work, however, has suggested the genus should be reclassified as a part of the Orobanchaceae family, along with several other parasitic angiosperms (Olmstead *et al.*, 2001, Oxelman *et al.*, 2005). In the United Kingdom there are two species within the genus *Rhinanthus*: *R. angustifolius* (greater yellow rattle) and *R. minor* (yellow rattle) both of which are facultative root hemiparasites. The latter of these two species can be further divided into six subspecies, of which only ssp. *minor* is spring flowering and is therefore easily distinguished from the others (Stace, 1997).

1.2.2 The Phenology and Basic Physiology of *Rhinanthus minor*

In the UK, *R. minor* seedlings begin to germinate in late February and early March and require a prolonged period of cold stratification to break dormancy. Seedling densities can be very high with up to 4000 seedlings m^{-2} being reported and typical high densities being in the region of 1000 plants m^{-2} (Westbury, 2004). Following germination, seedlings attempt to form haustorial connections with the roots of the surrounding vegetation. Contact between the root of the hemiparasite and that of a potential host will lead to the initialisation of haustoria, although some reports suggest that this process is far from specific, with *R. minor* attempting to form haustoria with bits of bark (Gibson and Watkinson, 1989). The haustorium initially swells around the potential host root, the developing endophyte within the haustoria then forms a penetrative peg that pierces the stele and xylem of the host. This endophyte contains secondary xylem vessels and therefore provides a direct xylem to xylem link between the host and hemiparasite (Cameron *et al.*, 2006). This allows the hemiparasite to remove large amounts of water (up to 20 % of the host's total water intake), together with various other solutes from the host plant's xylem stream (Jiang *et al.*, 2003, Jiang *et al.*, 2004b).

Following attachment, hemiparasites undergo a period of growth before producing flowers from late May onward. The flowers of *R. minor* are either insect pollinated or selfed (Westbury, 2004). Seed production generally overlaps with flowering, with both flowers and seeds being displayed simultaneously on the same plant (Westbury, 2004). Seed production can vary significantly, but importantly the seed produced each year provides the only source of propagules for the following season as the seeds of *R. minor* lose viability after just one year (Westbury, 2004).

1.2.3 Ecological Interactions of *Rhinanthus minor*

In common with many other parasitic plants, *R. minor* can form haustorial connections with multiple host species. Root excavation of *R. minor* showed that the hemiparasite formed haustoria with the roots of 50 different plant species from 18 families (Gibson and Watkinson, 1989). The performance of the hemiparasite varies considerably depending upon which host plant species it is attached to (Seel *et al.*, 1993a), either because of the host's ability to defend itself (Rumer *et al.*, 2007, Cameron *et al.*, 2006) or because of the variation in the amount nitrogen the hemiparasite can remove from its host (Seel *et al.*, 1993a). Importantly, however, *R. minor* is capable of attaching to multiple host plants simultaneously, and on average forms connections with four different plants (Gibson and Watkinson, 1989). This ability allows the hemiparasite potentially to take advantage of a broader range of solutes that may be supplied by multiple host species (Govier *et al.*, 1967).

Furthermore, in common with other parasitic plants, *R. minor* can have major impacts on the performance of its host (Cameron *et al.*, 2008, Jiang *et al.*, 2003) and on the composition of the plant community (Gibson and Watkinson, 1989, Pywell *et al.*, 2004). Because in the majority of cases the presence of *R. minor* leads to an increase in diversity, it has been suggested by numerous authors as a means of restoring the diversity of grassland ecosystems (Davies *et al.*, 1997, Westbury *et al.*, 2006, Bullock and Pywell, 2005). In addition to this, *R. minor* has also been shown to modify both nitrogen cycles and the balance of microbial communities (Bardgett *et al.*, 2006).

While the interactions between *R. minor* and its host plants and the plant community are relatively well understood, little is known about the interaction between this

hemiparasite and invertebrate herbivores (but see Bass, 2004). Furthermore, the hemiparasite *R. minor* is often present at very high densities (Westbury, 2004) and can form a considerable portion of the above ground biomass at certain times of the year (Davies *et al.*, 1997). It therefore represents an important potential food source for invertebrate herbivores; as such the interactions between *R. minor* and its invertebrate herbivores requires further investigation.

1.3 Aims of the thesis

The aims of this thesis are to investigate the interactions between the hemiparasite *Rhinanthus minor,* its host plants and its invertebrate herbivores, and to investigate some of the ecological factors which affect these interactions.

The thesis aims to address four key questions:

1. How does the density of the hemiparasite affect the composition of the plant community, the growth and survival of the hemiparasite and the levels of invertebrate herbivory it experiences?
2. What is the impact of host identity on the growth and reproductive output of the hemiparasite, and how do any consequent changes in *R. minor* performance affect its invertebrate herbivores?
3. What are the impacts of attaching to contrasting host plant species on the performance of *R. minor* and how do these effects, in turn, affect the hemiparasite's invertebrate herbivores?
4. Do increases in the levels of nutrients in the growing media and incidence of host plant damage affect the performance of *R. minor*, and do these changes

indirectly modify the interactions between the hemiparasite and its invertebrate herbivores?

These questions are addressed in the four following chapters. The sixth and final chapter discusses some broader questions relating to the ecology of *R. minor* and provides some ideas about the future work required in this area of research. I finish with some concluding comments on the importance of the work presented here.

Chapter 2. The Effects of Density on the Performance and Ecological Interactions of the Hemiparasite *Rhinanthus minor*

2.1 Abstract

The hemiparasite *Rhinanthus minor* is a common and important part of many grassland ecosystems. By negatively impacting on its hosts and reducing their competitive ability relative to non-hosts this hemiparasitic plant can have major effects on the plant community composition. Such impacts appear to be, at least partially, density dependent. Increasing density is however, likely to have profound effect on the performance of the hemiparasite itself. I investigate the impact of *R. minor* density on its performance, the levels of invertebrate herbivore damage the hemiparasite experiences, its mortality and its impacts on its host plants and surrounding plant community. This was achieved by establishing *R. minor* on replicated plots at four initial densities. In addition, a greenhouse experiment was conducted in which the hemiparasite was grown at a range of densities in pots with a single host plant.

Both experiments found that the mortality of the hemiparasites increased with increasing density. Furthermore, increasing the density of *R. minor* significantly reduced its growth, and, at high densities, reduced the total biomass of the hemiparasite. There was no effect of density on the levels of herbivore damage the hemiparasite experienced. Finally, and in agreement with several other studies, there was an increase in the impact on the surrounding vegetation with increasing hemiparasite density. There was a significant reduction in the percentage cover of

the grasses and an increase in bare ground. The results presented here suggest that the density of *R. minor* significantly affects its impacts on both the composition of the vegetation and its own performance. It appears however, that increasing density does not increase the vulnerability of the hemiparasite to herbivory.

Key words: Hemiparasite; *Rhinanthus minor*; invertebrate herbivores; herbivore damage; density dependence; survivorship: vegetation composition.

2.2 Introduction

Rhinanthus minor is a common hemiparasitic grassland plant which is distributed across much of the temperate northern hemisphere (Westbury, 2004). Like all other root parasites, *R. minor* forms attachments to the roots of other plants using specialised organs known as haustoria (Kuijt, 1969, Riopel and Timko, 1995). These organs, which contain secondary xylem, puncture the xylem stream of the host and thereby form a vascular link between the hemiparasite and host (Kuijt, 1969, Riopel and Timko, 1995, Cameron and Seel, 2007). Once this link is formed, the hemiparasite is able to remove water, together with a wide range of dissolved solutes, directly from the host's xylem stream (Jiang *et al.*, 2004b, Jiang *et al.*, 2003). The flow from host to hemiparasite is achieved because the hemiparasite maintains a high transpiration rate thereby creating a water potential lower than that of its host (Jiang *et al.*, 2003, Stewart and Press, 1990). This process can have a highly negative effect on the performance of *R. minor* hosts (Cameron *et al.*, 2008, Seel and Press, 1996), with a single hemiparasite removing as much as 20% of the host xylem sap (Jiang *et al.*, 2004b, Jiang *et al.*, 2003, Cameron and Seel, 2007).

R. minor has a very wide host range, forming haustorial attachments with as many as 50 host species from 18 separate families (Gibson and Watkinson, 1989). The negative effects of *R. minor* are not equal on all of the hemiparasite's potential hosts. For example, *Rhinanthus* spp. appear to affect grasses more negatively than either legumes or non-leguminous forbs (Seel and Press, 1996, Cameron *et al.*, 2008, Cameron *et al.*, 2009, Joshi *et al.*, 2000). Furthermore, the performance of *R. minor* is differentially affected by the identity of its host, tending to perform best on grasses and legumes (Seel *et al.*, 1993a, Cameron *et al.*, 2006, Seel and Press, 1993). One reason for this difference in *R. minor* performance on different hosts is the variation in the host plant's ability to actively defend its xylem stream from the developing haustoria of *R. minor* (Cameron *et al.*, 2006, Rumer *et al.*, 2007, Cameron and Seel, 2007).

These differential effects on the various components of the plant community suggests that hemiparasites can have a major impact on the productivity and composition of the vegetation, while also affecting nutrient cycling and microbial communities (Bardgett *et al.*, 2006, Ameloot *et al.*, 2005, Ameloot *et al.*, 2008, Davies *et al.*, 1997, Joshi *et al.*, 2000, Pywell *et al.*, 2004, Gibson and Watkinson, 1992). Numerous studies suggest that the primary impact of the parasite is a large reduction in primary productivity (Westbury and Dunnett, 2007, Davies *et al.*, 1997, Pywell *et al.*, 2004, Ameloot *et al.*, 2005, Joshi *et al.*, 2000), with average decreases in above ground biomass of between 40 and 60 % (Ameloot *et al.*, 2005). In most cases this decrease in productivity appears to lead to an increase in plant diversity (Davies *et al.*, 1997, Joshi *et al.*, 2000, Pywell *et al.*, 2004), although reductions in plant diversity have also been recorded in other cicumstances (Gibson and Watkinson, 1992).

The impact of *R. minor* on plant communities might be expected to vary with hemiparasite density, particularly given the wide range of densities at which it occurs. For example, Westbury (2004) reports natural mean densities of *R. minor* in the region of 750 plants m^{-2}, and seedling densities in excess of 4000 m^{-2} have been recorded (Westbury, 2004 and references therein), although more typical densities of flowering *R. minor* plants range between 100 and 200 m^{-2} (Westbury, 2004, Ameloot *et al.*, 2005). Furthermore, the impacts on the vegetation are often density dependent, with reductions in sward productivity increasing with hemiparasite density (Pywell *et al.*, 2004, Westbury and Dunnett, 2007, Davies *et al.*, 1997). Some studies suggest that such density-dependent effects reach a threshold at around 80 *R. minor* plants per m^2, beyond which no further impacts are observed (for example, Davies *et al.*, 1997), whereas other studies appear to find no such threshold (Ameloot *et al.*, 2005, Westbury and Dunnett, 2007). These variable results strongly suggest that the threshold of these density dependent interactions may vary from site to site, potentially reflecting variations in soil fertility (Davies and Graves, 2000) or in the species composition of the plant community.

In addition to the density-dependent effects of *R. minor* on the vegetation it seems likely that the parasite itself will suffer from intraspecific competition at increasing densities potentially resulting in self thinning. For example, Westbury and Dunnett (2007) observed that the fecundity of *R. minor* was significantly reduced at high sowing densities. It also seems likely that the impacts of intraspecific competition and the effects on intraspecific parasitism will result in a dramatic decrease in hemiparasite survivorship. For example, Prati *et al* (1997) demonstrated that intraspecific parasitism resulted in asymmetric competition in the hemiparasite *Rhinanthus serotinus*.

These increases in hemiparasite density are likely to result in changes in the levels of growth and chemical composition of the hemiparasite, and hence to damage by invertebrate herbivores (White, 1984, Huberty and Denno, 2004). Furthermore, the impacts of increased hemiparasite density on the surrounding vegetation may result in localised changes in the composition of the invertebrate community.

The aim of this study was to examine how the density of *R. minor* affects its performance and survivorship, and the levels of herbivore damage it experiences, using a combination of a field experiment and a greenhouse study. In the field experiment, plots of differing hemiparasite density were set up by thinning *R. minor* plants to four densities on an established area of grassland. This approach allowed me to assess the effect of hemiparasite density on its survivorship, final density and the level of herbivore damage it received. It also enabled me to assess the impact of *R. minor* density on the surrounding vegetation and to investigate whether hemiparasite density affected the number of xylem-feeding spittle bugs within the plots. In the greenhouse study, hemiparasites were grown with a single host plant (*Ononis repens*) at densities of 1, 3, 6 or 9 *R. minor* plants per pot. This approach allowed me to assess how *R. minor* density affects its own survivorship and performance (in terms of above ground biomass), and the performance of the host plant.

In summary, the current study aims to answer the following four questions:

1. How does increasing the density of *R. minor* impacts on its own survivorship and performance?
2. What effect does the density of *R. minor* have on the levels of herbivory it experiences?

3. How does increasing hemiparasite density affect the surrounding vegetation and the density of herbivores, specifically of spittle bug nymphs?
4. What impact does the density of *R. minor* have on the performance of its host plant?

The hypothesis being tested in each case was therefore:

1. Increasing the density of the hemiparasite will reduce its survivorship and performance due to increased competition for hosts and intraspecific parasitism.
2. Increased hemiparasite density will lead to increased stress levels in *R. minor* leading to higher levels of herbivore damage.
3. At higher hemiparasite density preferred hosts plants will show reduced performance leading to changes in the composition of the vegetation and this will also negatively impact on the herbivores of these hosts.
4. Individual host plants will show decreased performance with increasing hemiparasite density.

2.3 Method

2.3.1 Greenhouse Experiment

Rhinanthus minor seed was collected from Castle Hill National Nature Reserve in East Sussex (Grid ref: TQ 375 065) in late July 2007 and dried at ambient room temperature. *Ononis repens* (common restharrow) seeds were purchased from Emorsgate Seeds, Norfolk. All seeds were stored in sealed containers with silica gel at 4°C until required.

To initiate stratification, *R. minor* seeds were surface sterilised using 5% household bleach for 60 seconds and then washed four times in sterile water. Approximately 90 seeds were then placed onto 9cm petri dishes containing a damp layer of sterile Whatman glass fibre filter paper and capillary matting. Petri dishes were then sealed with parafilm and placed in a refrigerated room at 4°C for 84 days.

All plant material was grown in greenhouses at the University of Sussex, in which the temperature was maintained at 15-25°C with supplementary lighting (400W, high pressure sodium lamps) on a 16:8 light:dark regime. Plants were supplied with tap water *ad libitum*. *Ononis repens* plants were germinated on damp vermiculite 28 days after *R. minor* seed stratification had begun. After a further 14 days host plant seedlings were transplanted to 9cm pots containing six parts sand to one part John Innes No. 2 potting compost. 42 days later, ten germinating *R. minor* seedlings were transplanted into each pot. 120 pots were then assigned to one of four treatments, in which target densities were one host plant and either, one, three, six or nine *R. minor* plants. Pots were then arranged into 30 randomized blocks all containing one of each of the four treatments. Hemiparasite seedlings were thinned to the required density when the majority showed signs of attachment (Klaren and Janssen, 1978), which was approximately 21 days after *R. minor* seedlings were transplanted.

The above ground biomass of both the host plant and the hemiparasite were harvested when the first hemiparasites showed signs of senescence (approximately 100 days after they were introduced). All plant material was then dried at 60°C for two days and then weighed.

2.3.2 Field Experiment

The field experiment was conducted on the University of Sussex Field Trials Plot, located within the campus of the University of Sussex (Grid ref: TQ 348 096). The vegetation is largely dominated by four species of grass (*Holcus lanatus*, *Festuca* spp, *Dactylis glomerata* and *Agrostis stolonifera*) with a much smaller proportion of non-leguminous forbs (mainly: *Plantago lanceolata*, *Ranunculus* spp. and *Succisa pratensis*) and legumes (*Medicago lupulina*, *Lotus corniculatus* and *Trifolium repens*). An area of approximately 200 m^2 was mown in the autumn of 2007 to prepare the site for the experiment. Ten experimental blocks each containing four $0.5m^2$ plots with 0.5m borders separating each plot where then permanently marked out with stakes. These plots were then raked in order to remove any excess thatch that would inhibit *R. minor* germination.

In early December 2007, 1g of *R. minor* seed (approximately 500 seeds) was sown onto each plot. Plots were then randomly assigned to one of four *R. minor* density treatments so that each block contained one replicate of each treatment. The treatment densities were: 960, 720, 480 and 240 plants m^{-2}. *R. minor* seedlings began to emerge early in April 2008 and were thinned to the desired density at the beginning of May when they had produced their first true leaves but were yet to show signs of attachment (Klaren and Janssen, 1978).

On 19th May 2008 five plants from each plot were tagged using coloured cotton thread. Herbivore damage of the 10 basal leaves of each plant was estimated by eye as a percentage of leaf area removed. Damage estimates where then repeated on a further four occasions between 19th May and 30th June, always from the same five tagged plants. Because of the high leaf turnover, no attempt was made to make

comparisons between dates (i.e. damage rates) as very few leaves were present from one survey to the next. The mean damage levels from each plot were used in the final analysis (to avoid pseudo-replication), and sufficient numbers of plants survived until the final survey for this to be calculated from all plots except in two cases (in the 960 and 480 m^{-2} treatments).

To gain an insight into the potential impact of *R. minor* density on generalist invertebrate herbivores the number of spittle bugs (*Neophilaenus lineatus* and *Philaenus spumarius*) feeding on the plots was assessed on three separate occasions between 28th May and 11th June. Because both species form conspicuous "spittle" masses and are relatively sedentary, counts could be made with minimal disturbance to the plot. Furthermore, *N lineatus* and *P. spumarius* feed exclusively on grasses and forbs, respectively (Weaver and King, 1954, Whittaker, 1965) and therefore gave some insight into any potential differential impacts of parasite density on these plant groups and the impacts on other herbivores.

On 26th June 2008 a vegetation survey was undertaken to assess how *R. minor* density affected the composition of the vegetation. This was achieved by visual estimation of the percentage cover of the three main functional groups (grasses, legumes and non-leguminous forbs), the percentage of bare ground and the percentage cover of the hemiparasite *R. minor*. Finally, the total number of *R. minor* plants in each plot was counted on five occasions between 2nd June and 4th July following the initial thinning.

2.3.3 Statistical Analysis

All statistical analyses were carried using Minitab 15 for Windows (Minitab, Inc). Whenever possible the data were analysed using two-way analysis of variance

(ANOVA) with density treatment as the main factor; and block included as a random factor (block was excluded from the final analysis if it was not significant). When necessary, the data were transformed to meet the assumptions of the test and in all cases percentage data were square-root arcsine transformed prior to analysis. Whenever it was not possible to meet the assumptions of the test analogous non-parametric tests were utilized.

2.4 Results

2.4.1 Greenhouse Experiment

There was a strong positive relationship between the density of the hemiparasites and their percentage mortality (Kruskal-Wallis; H_3 = 12.09, P = 0.007). Pots that contained three *Rhinanthus minor* plants had the lowest mean percentage mortality rate, and those with nine hemiparasites had the highest (Figure 1). Therefore, the final number of complete replicates (i.e. those with all the *Rhinanthus minor* plants still present at the end of the experiment) for the pots containing one, three, six or nine hemiparasites was reduced from 30 to 22, 22, 15 and 6, respectively.

R. minor showed a significant decrease in mean above ground biomass as the density of plants increased ($F_{3,61}$ = 3.75, P = 0.015), with hemiparasites grown without conspecifics producing higher biomass than any other treatment and significantly higher biomass than parasites grown with eight other conspecifics (Figure 2). The mean cumulative biomass of hemiparasites per pot (the yield per pot) was also affected by the density of hemiparasites ($F_{3,61}$ = 2.89, P = 0.043), with pots containing six hemiparasites producing significantly more biomass than those

containing either one or three *R. minor* plants (Figure 3). Pots containing nine hemiparasites appeared to show only a slight and non-significant reduction in total hemiparasite biomass, although this may be a product of the relatively low number of replicates for this treatment (Figure 3). The density of the hemiparasites had no effect on the above ground biomass of the host plant *Ononis repens* (ANOVA; $F_{3,62} = 0.67$, $P = 0.575$) (Figure 4).

2.4.2 Field experiment

The initial density of *R. minor* had a strong negative effect on the percentage cover of grasses in the experimental plots (two-way ANOVA: initial density, $F_{3,27} = 4.07$, $P = 0.017$; block, $F_{9,27} = 5.08$, $P < 0.001$) (Figure 5 and Table 1), with plots initially containing 960 *R. minor* plants per m^{-2} having significantly lower percentage cover of grasses than plots with 240 *R. minor* plant per m^{-2}. The other functional groups (legumes and non-leguminous forbs) and the percentage of bare ground were not significantly affected by the *R. minor* density treatments (two-way ANOVA: legumes, initial density, $F_{3,27} = 1.48$, $P = 0.243$; block, $F_{9,27} = 3.55$, $P = 0.005$; non-leguminous forbs, initial density, $F_{3,27} = 0.70$, $P = 0.560$; block, $F_{9,27} = 1.99$, $P = 0.081$; bare ground, initial density, $F_{3,27} = 0.80$, $P = 0.504$; block, $F_{9,27} = 1.08$, $P = 0.409$) (Figure 5). There was a noticeable, but non-significant increase in percentage of bare ground as density of the hemiparasites increased, presumably due to the reduction in grass biomass. The percentage cover of *R. minor* was significantly affected by its initial density (two-way ANOVA; initial density, $F_{3,27} = 9.45$, $P < 0.001$; block, $F_{9,27} = 15.36$, $P < 0.001$) (Figure 5). At the time of the vegetation survey there was still a significant difference in the density of *R. minor* overall (two-way ANOVA; initial density, $F_{3,27} = 26.81$, $P < 0.001$; block, $F_{9,27} = 2.02$, $P = 0.076$). *Post-hoc* analyses, however, revealed that the two highest densities were no

longer significantly different from each other (the other comparisons were different at the P=0.05 level).

The initial density of *R. minor* had a highly significant effect on subsequent percentage mortality ($F_{3,36}$ = 21.54, P < 0.001). This was highest in plots with the highest initial density and *vice versa* (Figure 6). Interestingly, despite the large range of initial densities, there was no significant difference in the final densities of the hemiparasites (two-way ANOVA; initial density, $F_{3, 27}$ = 1.31, P = 0.291; block, $F_{9, 27}$ = 1.10, P = 0.393) (Figure 7), with the final mean densities for all treatments varying by less than 40 *R. minor* plants m^{-2}.

The numbers of both species of spittle bug were not significantly affected at any stage of the experiment by the initial density of the hemiparasites (see Supplementary Material). Initial *R. minor* density had no significant effect on the amount of damage that plants received at five dates during the experiment (Figure 8 and Supplementary Material).

2.5 Discussion

Numerous studies have shown that the hemiparasite *Rhinanthus minor* can have major impacts on grassland ecosystems (Bardgett *et al.*, 2006, Ameloot *et al.*, 2005, Ameloot *et al.*, 2008, Davies *et al.*, 1997, Joshi *et al.*, 2000, Pywell *et al.*, 2004, Gibson and Watkinson, 1992), and in many cases these effects appear to be density dependent, with the hemiparasite having increasingly dramatic effects at high densities (Pywell *et al.*, 2004, Joshi *et al.*, 2000, Westbury and Dunnett, 2007, Davies *et al.*, 1997). This study has also shown that the impacts of *R. minor* on the

composition of the vegetation are highly density-dependent. In addition to this, it has demonstrated that the effects of *R. minor* on plant communities are found across a very large range of hemiparasite densities. Furthermore, these impacts on the vegetation also appear to occur very rapidly, leading to significant differences in the proportion of grass cover within approximately two months of *R. minor* emergence.

In agreement with the majority of other studies, I found that the main impact of the hemiparasite was on the grasses (Seel and Press, 1996, Cameron *et al.*, 2008, Cameron *et al.*, 2009, Joshi *et al.*, 2000). This is presumably because of the inability of this group of plants to defend themselves from the developing haustoria of *R. minor* (Cameron *et al.*, 2006, Rumer *et al.*, 2007, Cameron and Seel, 2007). In contrast to the results of some studies (for example Pywell *et al.*, 2004) there were no significant differences between treatments in the percentage cover of the non-leguminous forbs, perhaps due to the relatively short time scale of this study. However, the marked (although non-significant) increases in the percentage of bare ground cover with increasing hemiparasite density may well have resulted in increases in the percentage cover of the other functional plant groups due to competitive release (Pennings and Callaway, 1996, Callaway and Pennings, 1998, Gibson and Watkinson, 1991) . Furthermore, increases in the amount of bare ground may well have resulted in enhanced seedling recruitment, an effect which has been observed previously (Joshi *et al.*, 2000).

The density of the hemiparasite had no impact on the percentage cover of legumes, despite numerous reports that legumes are particularly good hosts for *R. minor* (Seel *et al.*, 1993a, Seel and Press, 1993). Furthermore, hemiparasite density had no noticeable effect on the biomass of the legume, *Ononis repens*, used in the

greenhouse experiment. This has also been observed previously by Davies *et al* (1997) and may be because the legumes are not limited by nitrogen and can therefore sustain the parasite with no noticeable reduction of biomass. In addition, the *ad libitum* water regime used in the greenhouse experiment and the slow draining substrate at the field site may mean that there was no water stress imposed on plants, thus relieving another of the negative effects imposed on the host by this hemiparasite (Jiang *et al.*, 2003). It is also possible that while biomass and percentage cover showed no response to hemiparasite density, other aspects of their ecology, such as levels of chemical defences (Puustinen and Mutikainen, 2001) and fecundity, may well have been negatively affected.

The density of spittle bug nymphs, whether they were the grass-feeding (*Neophilaenus lineatus*) or forb-feeding (*Philaenus spumarius*) showed no responses to the density of the hemiparasite. This is perhaps surprising given the large reduction in grass cover within these plots, and because both hemiparasites and spittle bugs feed on the xylem stream of their host and so might be expected to compete. Indeed, Bass (2004) observed a significant increase in the mortality of *Neophileanus lineatus* feeding on grasses that were grown with *R. minor*. Negative indirect impacts of this hemiparasite on the performance of invertebrate herbivores feeding on its host plant have been reported elsewhere (Puustinen and Mutikainen, 2001, Bass, 2004), and potentially have major implications for the composition of invertebrate herbivore communities.

Unsurprisingly, increasing the density of the *R. minor* had an increasingly negative effect on its own survivorship in both the field and greenhouse experiment. This resulted in such high mortality rates that there was no significant difference in the

density of parasites at the end of the field experiment despite the highest densities being initially four times higher than the lowest. This strongly suggests that the maximum carrying capacity for *R. minor* within this particular location, at least during the first year of its presence, is approximately 165 plants m^{-2}, which is well within the range reported elsewhere (Westbury, 2004, Ameloot *et al.*, 2005). Interestingly, the highest overall yield of *R. minor* in my greenhouse experiment occurred in pots containing six hemiparasites, with densities above this showing reduced yield. This decline in overall yield at very high densities was also observed by Westbury and Dunnett (2007) and Davies *et al* (1997), and again suggests a threshold over which the performance of the hemiparasites is greatly impaired. While total yield tended to increase up to a threshold, the performance of individual plants decreased with increasing hemiparasite density. It seems likely that the reduced individual biomass of hemiparasites is due to increased competition for resources, and because of the impact of intraspecific parasitism. The reduction in plant biomass may also lead to a reduction in reproductive output for the hemiparasite, and indeed Westbury and Dunnett (2007) suggest that increases in competition lead to a decrease in fecundity.

Despite the effects of density on the survivorship and performance of the hemiparasite, my investigations into the impacts of hemiparasite density on the level of herbivore damage revealed no significant effects. Clearly the impacts of hemiparasite performance were insufficient to influence herbivore preference in this system.

An alternative hypothesis is that host identity has a greater impact on the hemiparasite's herbivores than hemiparasite density, possibly because host identity

causes larger changes in the hemiparasite's chemical composition (e.g. Govier *et al.*, 1967, Marvier, 1996). The site of the field experiment was dominated by only a few grass species, and the percentage cover of the other group of preferred hosts, the legumes (as demonstrated by Seel *et al.*, 1993a, Seel and Press, 1993), was very low, suggesting that most *R. minor* plants were attached to only few host species. Numerous studies have demonstrated that the identity of the host plant can have major differential impacts on the performance of *R.minor* (Seel *et al.*, 1993a, Cameron *et al.*, 2006, Seel and Press, 1993). Furthermore, there is a large body of evidence demonstrating that the identity of a hemiparasite's host can have major effects on their interactions with invertebrate herbivores (Adler, 2000, Adler *et al.*, 2001, Adler, 2002, Marvier, 1996, Marvier, 1998, Lehtonen *et al.*, 2005).

Overall, the current study has demonstrated that the density of *R. minor* can have an important impact on the composition of the surrounding vegetation at an extremely wide range of densities. The severe impacts of this hemiparasite at high densities are, however, likely to have increasing effects on other groups of organisms known to interact with *R. minor*, such as mycorrhiza (Davies and Graves, 1998) and some invertebrate herbivores (Bass, 2004, Puustinen and Mutikainen, 2001). I have also demonstrated that increased density of this hemiparasite leads to an increase in mortality and a reduction in its onwn performance, which is likely to have major implications for its population dynamics and hence for numerous other organisms within the community.

2.6 Tables

Table 1. The impact of different *Rhinanthus minor* densities on the composition of the surrounding vegetation. The hemiparasite starting densities were either: 960, 720, 480 or 240 *R. minor* plants m^{-2}. Two-way ANOVA, all treatments n =10, all data were square-root arcsine transformed prior to analysis.

Element of vegetation	Factor	*df*	*F*	*P*
Grasses	*R. minor density*	3	4.07	0.017
	Block	9	5.08	< 0.001
Legumes	*R. minor density*	3	1.48	0.243
	Block	9	3.55	0.005
Other forbs	*R. minor density*	3	0.70	0.560
	Block	9	1.99	0.081
Bare ground	*R. minor density*	3	0.80	0.504
	Block	9	1.08	0.409
R. minor	*R. minor density*	3	9.45	< 0.001
	Block	9	15.36	< 0.001

2.7 Figures

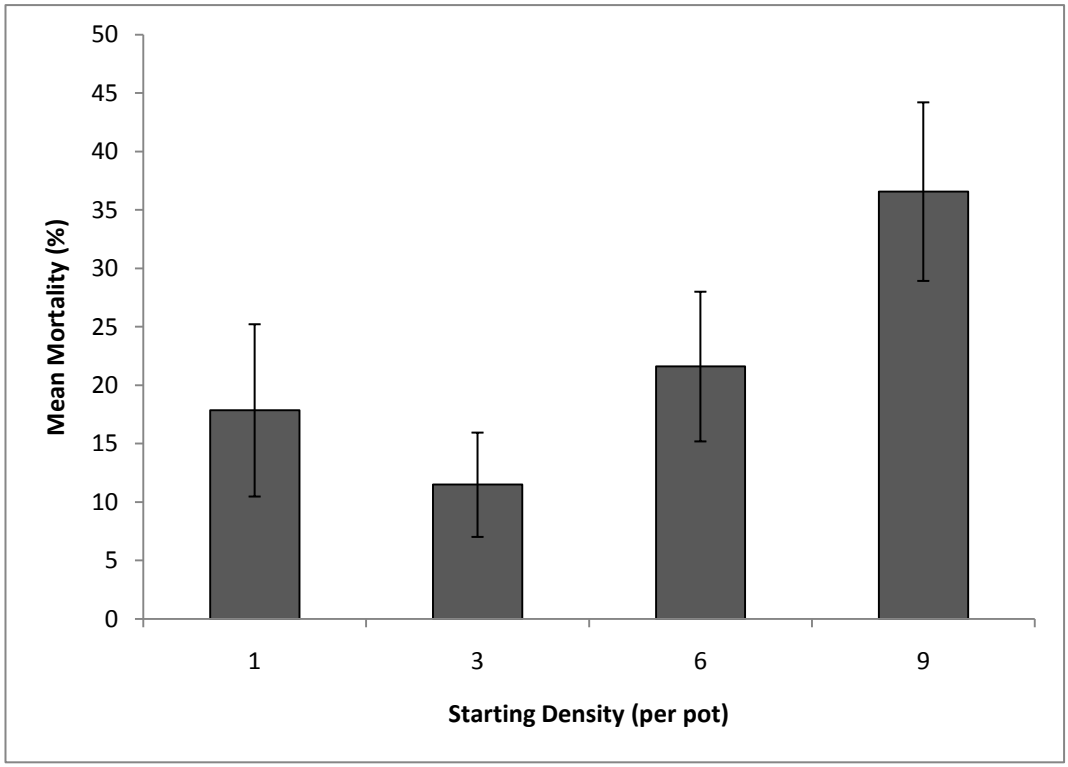

Figure 1. The mean percentage mortality of *Rhinanthus minor* grown with the host plant *Ononis repens* and at a density of 1, 3, 6 or 9 hemiparasites per pot. Error bars show ± 1 SEM. Kruskal-Wallis; $H_9 = 12.09$, $P = 0.007$.

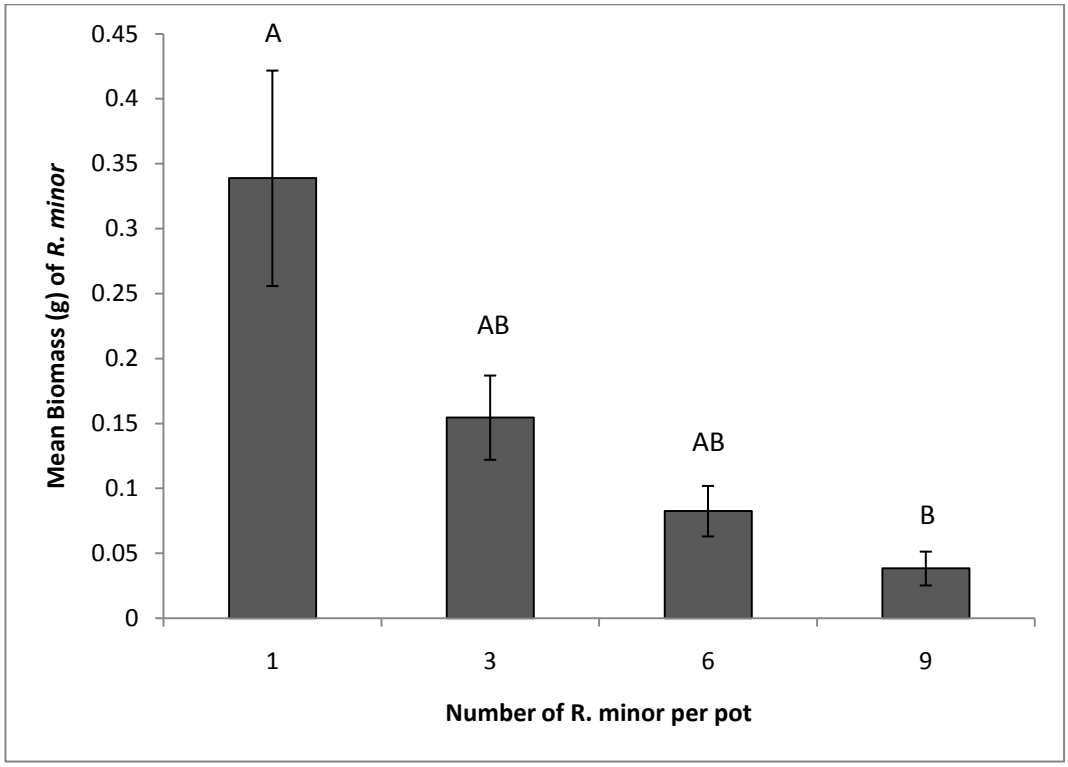

Figure 2. The mean above ground biomass (g) of *Rhinanthus minor* grown with the host plant *Ononis repens* at a density of 1, 3, 6 or 9 hemiparasites per pot. Dependent variable for density treatment 3, 6 and 9 was chosen at random from each pot. Error bars show ± 1SEM. One-way ANOVA; $F_{3,61}$ = 3.75, P = 0.015. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis P=0.05). Data w ere $\log_{10}$ transformed prior to the analysis. Replicate numbers for each treatment were 22, 22, 15 and 6, respectively.

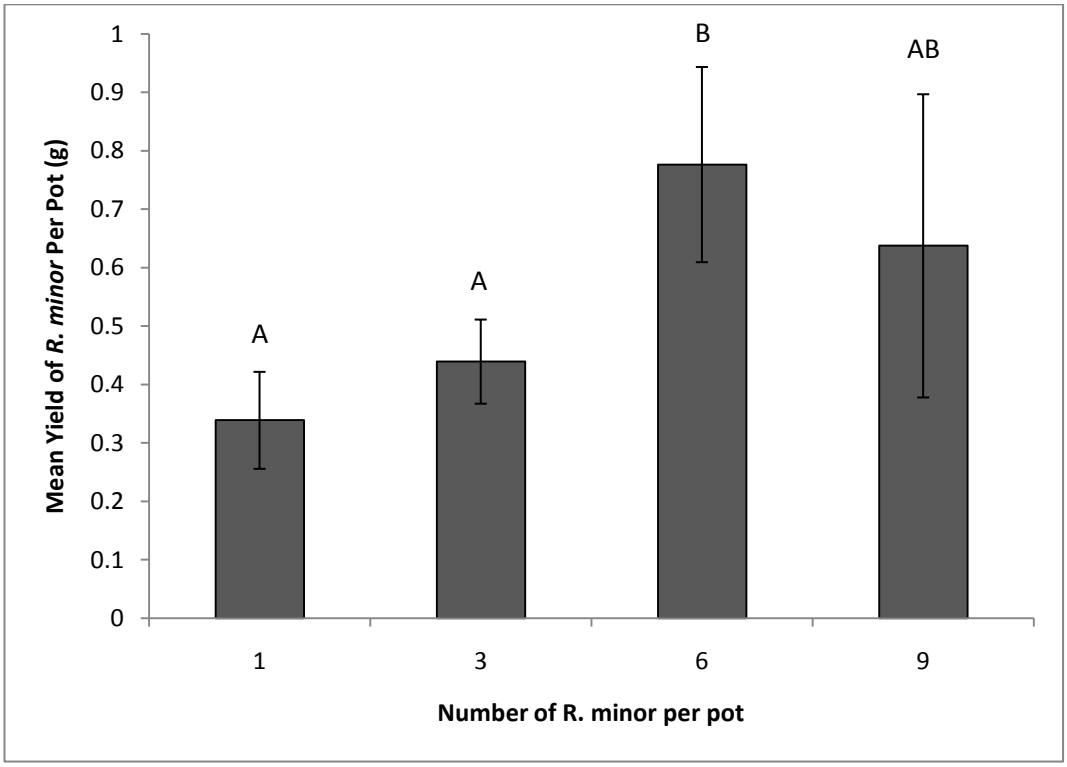

Figure 3. The mean yield (g) of *Rhinanthus minor* per pot when grown with the host plant *Ononis repens* at a density of either 1, 3, 6 or 9 hemiparasites in each pot. Error bars show ± 1SEM. One-way ANOVA; $F_{3,61}$ = 2.89, P = 0.043). Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis P=0.05). Data were $\log_{10}$ transformed prior to the analysis. Replicate numbers for each treatment were 22, 22, 15 and 6, respectively.

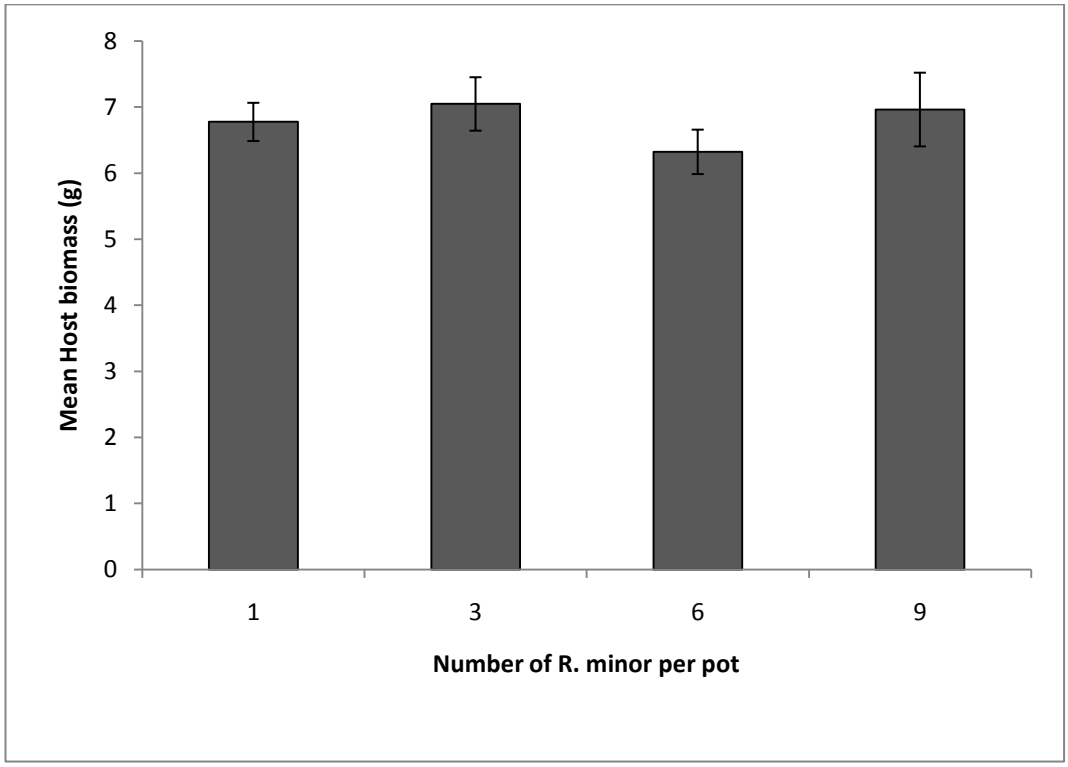

Figure 4. The mean biomass (g) of the host plants *Ononis repens* grown with either 1, 3, 6 or 9 *Rhinanthus minor* plants. Error bars show ± 1SEM. One-way ANOVA; $F_{3,62} = 0.67$, $P = 0.575$**).** Replicate numbers for each treatment were 22, 23, 15 and 6, respectively.

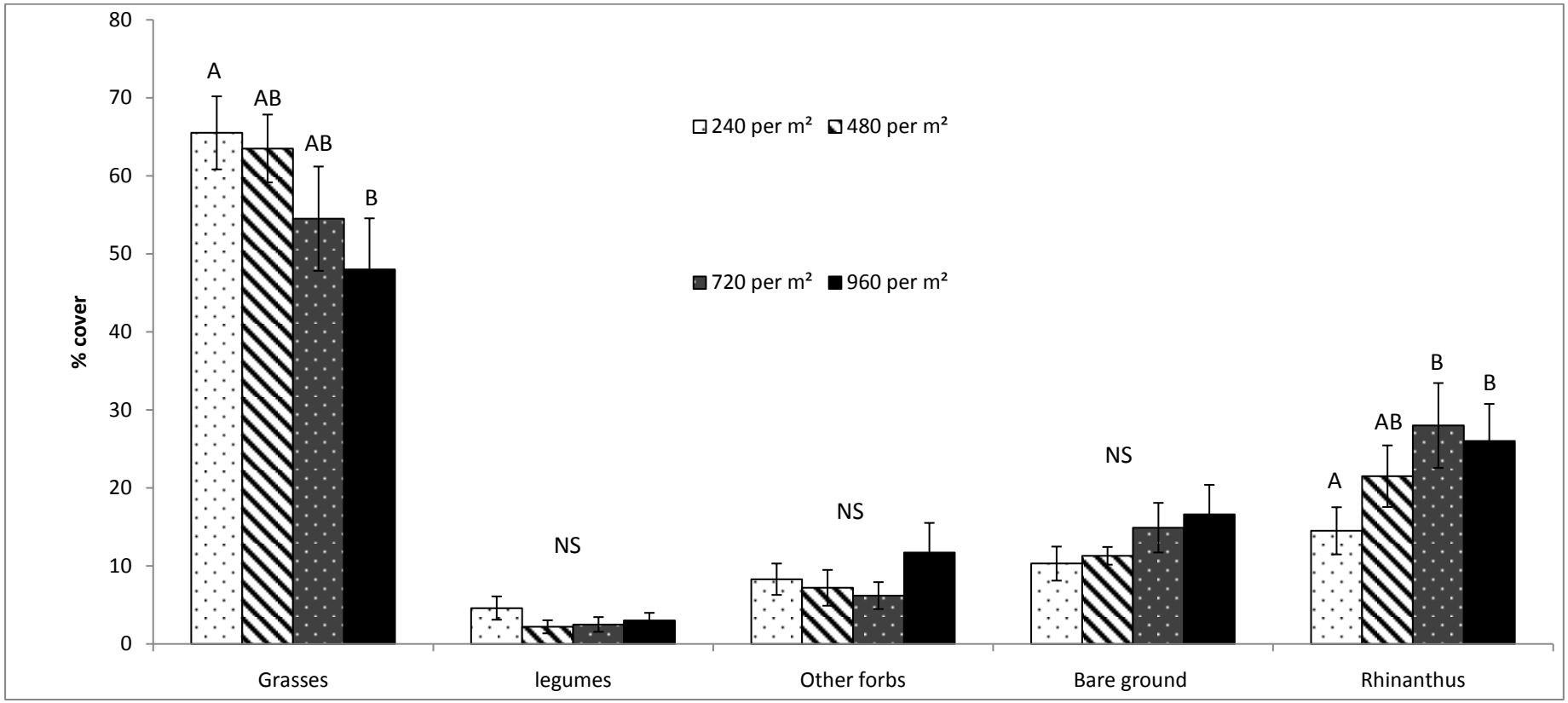

Figure 5. The mean percentage cover of the vegetation in plots containing the hemiparasites *Rhinanthus minor* at initial densities of 960, 720, 480 and 240 plants per m^{-2}. Error bars show ± 1SEM. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis P=0.05). Results from ANOVA can be found in Table 1. Data were Square-root arcsine transformed prior to analysis.

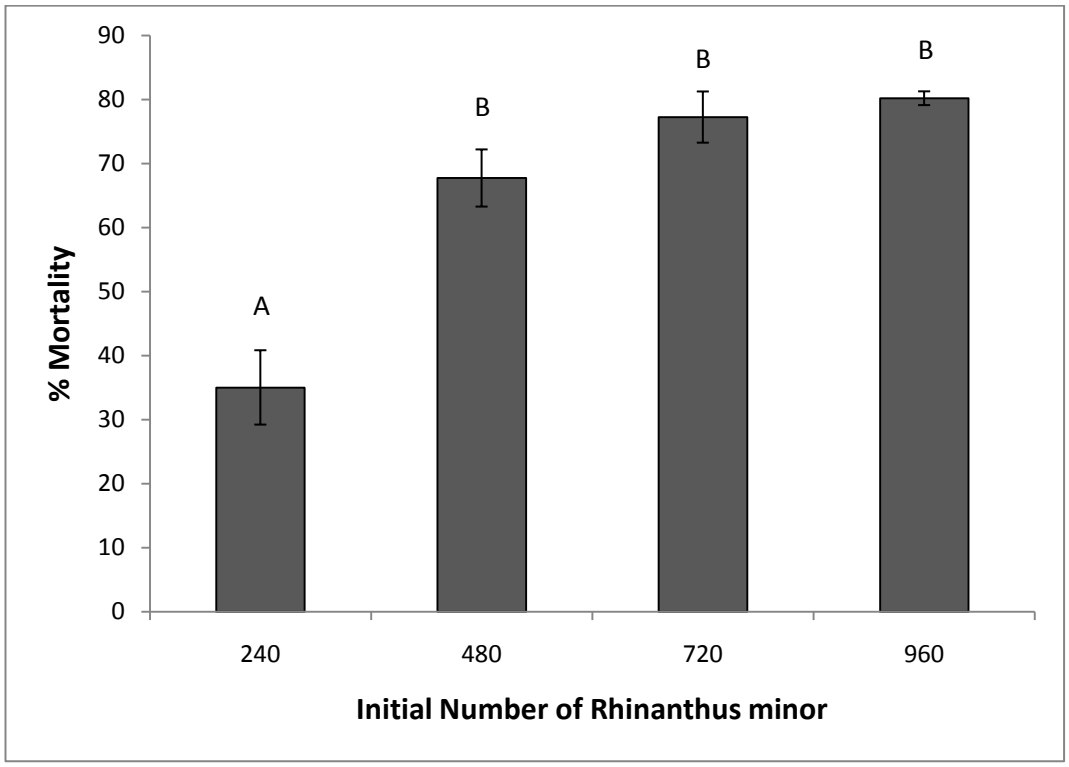

Figure 6. The mean percentage mortality of *Rhinanthus minor* plants grown in plots containing 960, 720, 480 or 240 hemiparasites m^{-2}. Error bars show ± 1SEM. One-way ANOVA; $F_{3,36} = 21.54$, $P < 0.001$. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis $P=0.05$). Data were square-root arcsine transformed prior to the analysis.

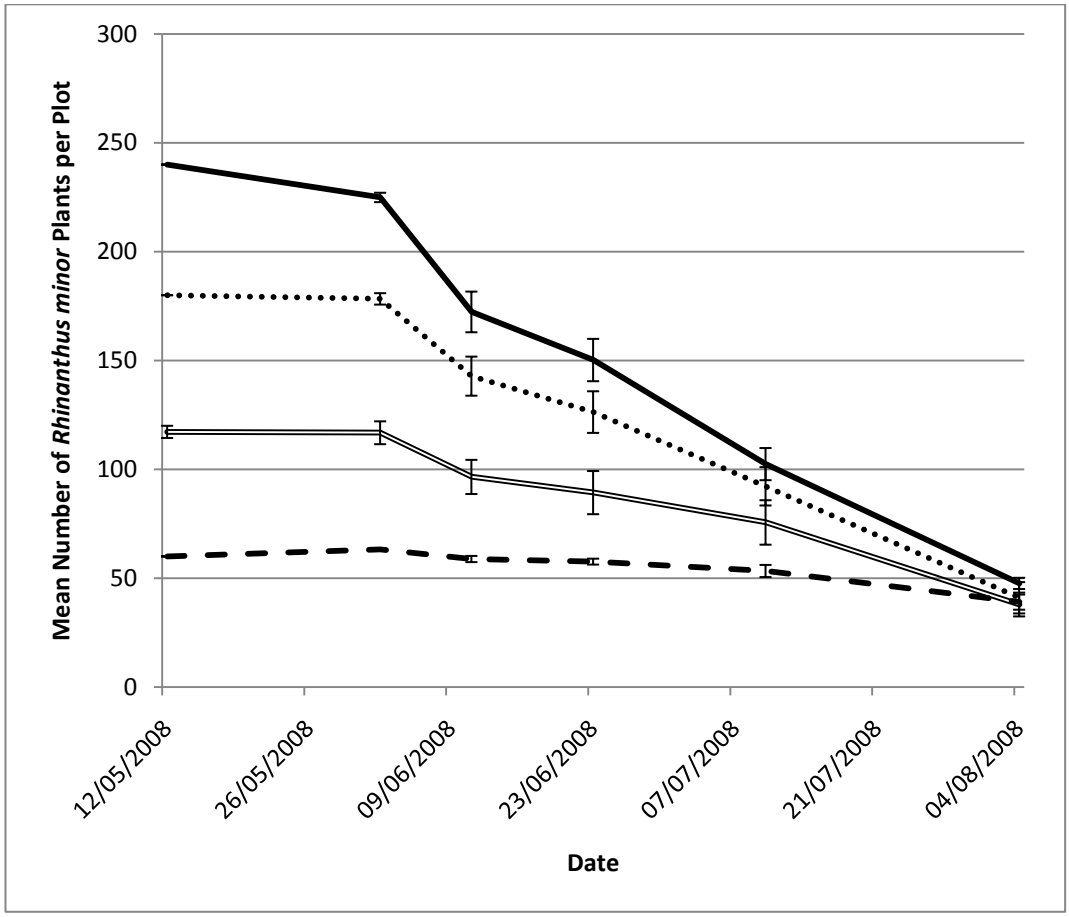

Figure 7. The mean number of *Rhinanthus minor* plants per plot throughout the experimental period. Initial plot densities were 240, 180, 120 or 60 hemiparasites per plot, with final mean plot densities for these treatment of 47.6, 41.0, 37.9 and 39.0 respectively. There was no significant difference in the final density (two-way ANOVA; initial density $F_{3,27} = 1.31$, $P = 0.291$; block, $F_{9,27} = 1.10$, $P = 0.393$). Data were $\log_{10}$ transformed prior to the analysis.

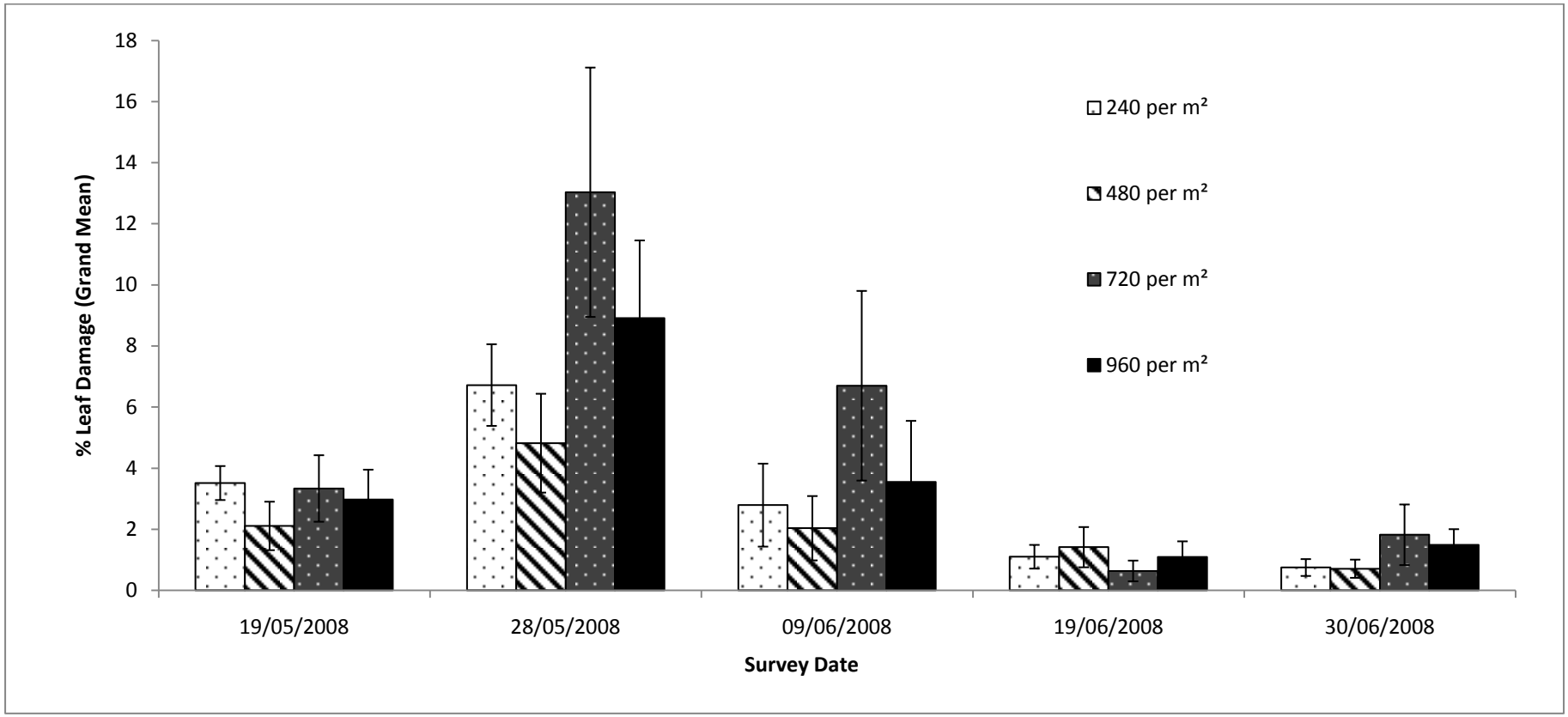

Figure 8. The grand mean of percentage herbivore damage experienced by *Rhinanthus minor* plants grown in plots containing 960, 720, 480 or 240 hemiparasites m^{-2}. Error bars show ± 1SEM. Results from ANOVA can be found in table 2 of the supplementary material. Data were Square-root arcsine transformed prior to analysis.

2.8 Supplementary Material

Table 2. The impact of different *Rhinanthus minor* densities on the number of spittle bug nymphs within the experimental plots. The hemiparasite starting densities were 960, 720, 480 or 240 *R. minor* plants m^{-2}. Analysis was carried out using a Friedman test (*S*= Friedman statistic), all treatments n = 10.

Survey Date	Spittle Bug Species	Factor	*df*	*S*	*P*
19/05/2008	*Philaenus spumarius*	Density	3	3.17	0.366
		Block	9	20.05	0.018
	Neophilaenus lineatus	Density	3	1.04	0.791
		Block	9	7.23	6.13
28/05/2008	*Philaenus spumarius*	Density	3	4.15	0.245
		Block	9	13.81	0.129
	Neophilaenus lineatus	Density	3	1.48	0.686
		Block	9	8.40	0.494
09/06/2008	*Philaenus spumarius*	Density	3	0.66	0.883
		Block	9	8.13	0.521
	Neophilaenus lineatus	Density	3	2.44	0.487
		Block	9	10.72	0.295

Table 2. The impact of different *Rhinanthus minor* densities on the level of herbivore damage (percentage leaf damage) experienced by tagged *R. minor* plants. In all cases grand means were used in order to avoid pseudo-replication. The hemiparasite starting density treatments were 960, 720, 480 or 240 *R. minor* plants per m^{-2}. Two-way ANOVA, all treatment n = 10, all data were square-root arcsine transformed prior to the analysis. Final date was analysed using a Kruskal-Wallis test because of missing values and non-normal data.

Date	Factor	*df*	*F*	*P*
19/05/2008	Density	3	0.91	0.449
Error *df* = 27	Block	9	0.43	0.907
28/05/2008	Density	3	2.12	0.121
Error *df* = 27	Block	9	2.53	0.030
09/06/2008	Density	3	1.73	0.184
Error *df* = 27	Block	9	2.15	0.060
19/06/2008	Density	3	0.79	0.511
Error *df* = 27	Block	9	0.86	0.570
30/06/2008	Density	3	*H*=1.11	0.774

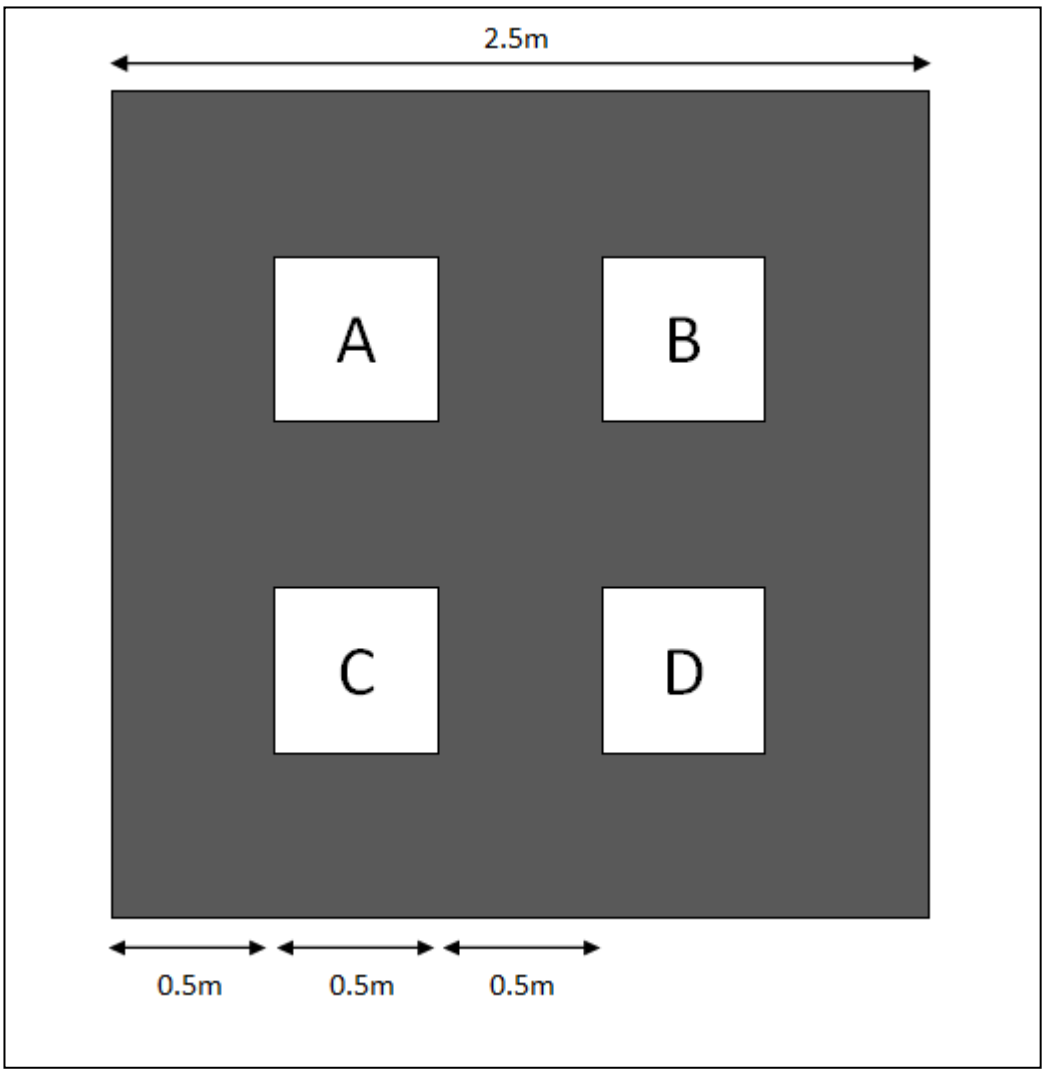

Figure 9. Typical layout of the treatments within each experimental block.

Chapter 3. The Effect of Host Identity on the Performance of the Hemiparasite Rhinanthus minor and its Invertebrate Herbivores

3.1 Abstract

The hemiparasite *Rhinanthus minor* is an important component of many grassland ecosystems and can have major impacts on the composition of grasslands communities, in part due to its ability to form functional parasitic associations with some plants and not others. Attaching to different host plants is known to have major and differential impacts on the growth reproduction and chemical composition of *R.minor*, but very little is known about how these effects might impact upon its invertebrate herbivores. The aim of this study was to investigate the effects of host identity on the performance of the hemiparasite and on its invertebrate herbivores. Phytophagous invertebrates from three separate feeding guilds were used in order to assess whether there are consistent herbivore responses to any effects of host identity.

There were very strong effects of host plant on the performance of both the hemiparasite and its invertebrate herbivores. Hemiparasites attained the greatest biomass if attached to the leguminous host plants *Ononis repens* and *Lotus corniculatus*, and the least when grown with *Plantago lanceolata*. Patterns in the performance of the invertebrate herbivores feeding on *R. minor* were not consistent across the three feeding guilds. In particular, *R. minor* plants grown with *L. corniculatus* resulted in the poorest performance for aphids and snails, and the best performance for froghoppers. For the majority of the other host plants tested,

however, the biomass of the hemiparasite appears to be a good predictor of the performance of the invertebrate herbivore, regardless of the feeding guild. The results presented in this study suggest that *R. minor* is a highly variable food source for invertebrate herbivores, with the identity of the host plant having a strong indirect effect on the hemiparasite and its herbivores. This variability may help to explain the paucity of specialist herbivores associated with it.

Key Words: Hemiparasite; *Rhinanthus minor*; host parasite interaction; invertebrate herbivores; C:N ratio; indirect interaction.

3.2 Introduction

Hemiparasitic plants are important components of many terrestrial ecosystems and can have major impacts on their ecological communities (Irving and Cameron, 2009, Phoenix and Press, 2005, Press and Phoenix, 2005). When present they can modify the plant community structure (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Davies *et al.*, 1997, Ameloot *et al.*, 2005, Pennings and Callaway, 1996) and soil microbial communities (Bardgett *et al.*, 2006), and also affect nutrient cycling (Bardgett *et al.*, 2006, Ameloot *et al.*, 2008, Quested *et al.*, 2002, Quested *et al.*, 2003a, Quested, 2008).

Hemiparasites acquire at least part of their nutritional requirements by attaching to the vascular systems of other plants via specialist organs known as haustoria (Kuijt, 1969, Riopel and Timko, 1995). The majority of hemiparasitic plants are capable of forming these attachments with a wide range of host plants (Kuijt, 1969), which can have major implications for their herbivores. For example, work on numerous

species of hemiparasite has demonstrated that the identity of the host plant can have a major impact on their growth rate (and thereby production of available biomass) and performance (Seel *et al.*, 1993a, Ahonen *et al.*, 2006, Seel and Press, 1993, Cameron *et al.*, 2006, Marvier, 1996, Press *et al.*, 1993). Furthermore, the transfer of secondary metabolites from some host plants to the hemiparasite can deter invertebrate herbivores (Adler *et al.*, 2001, Adler, 2000, Adler, 2002, Adler, 2003) and decrease their growth and performance (Marvier, 1996, Marvier, 1998), although the host's toxicity is not always a good predictor of the palatability of the hemiparasite (Schadler *et al.*, 2005). In addition to this, the nutritional quality of the host (Puustinen and Mutikainen, 2001), and the amount of nitrogen removed from the host (Seel *et al.*, 1993a, Press *et al.*, 1993) may also affect the performance of the hemiparasite's herbivores (Mattson, 1980, White, 1984).

Rhinanthus minor is a common hemiparasite of meadows and grassland and where it can constitute as much as 8% of the above ground standing biomass (Davies *et al.*, 1997). In common with many other hemiparasites, it can have major impacts on the composition of the vegetation (Pywell *et al.*, 2004, Bullock and Pywell, 2005, Bullock *et al.*, 2007, Gibson and Watkinson, 1992, Ameloot *et al.*, 2005) and below ground ecology (Bardgett *et al.*, 2006, Ameloot *et al.*, 2008). While it is clear that this plant forms an important component of many ecosystems, its impact on invertebrate communities remain poorly understood and its suitability as a food source for invertebrate herbivores has received little or no attention.

R. minor has been reported to form haustorial connections with around 50 different plant species from 18 families (Gibson and Watkinson, 1989). As with many hemiparasites, its growth and fecundity varies widely depending on the species to

which it is attached (Seel *et al.*, 1993a, Cameron *et al.*, 2006). Recent work on the haustorial interface suggests that the reasons for this variation in performance is the ability of many non-leguminous forbs to defend their xylem stream from the hemiparasite (Cameron *et al.*, 2006, Rumer *et al.*, 2007, Cameron and Seel, 2007). It seems likely that such interactions will dramatically change the quality of *R. minor* as a food source for invertebrate herbivores by increasing stress levels within the plant (White, 1984, Larsson, 1989, Huberty and Denno, 2004) and decreasing the amount of available nutrients (such as nitrogen) (Mattson, 1980).

In addition to the variation caused by attachment success, it is likely that the types and quantities of solutes passed from the host to the hemiparasite will not only affect its own growth, but also the performance of its invertebrate herbivores. This could be due to the effects of increased nitrogen availability to the hemiparasites (Seel *et al.*, 1993a, Press, 1995). For example, leguminous hosts may provide the hemiparasite with increased levels of nitrogen compared to other hosts such as grasses or non-leguminous forbs. Alternatively, some host plants provide *R. minor* with a range of secondary metabolites which it may utilise for its own defence (Atsatt, 1977). The complexity of these interactions potentially makes *R. minor* an extremely variable and potentially unpredictable source of food for phytophagous invertebrates. The primary aim of this study is to investigate how the effects of host identity on the hemiparasite *R. minor* impacts on the performance of invertebrate herbivores feeding on it.

It is important to acknowledge that the mode by which invertebrates acquire their food (i.e. their feeding guild) can have important and differential impacts on their performance (Cockfield, 1988, Larsson, 1989, Huberty and Denno, 2004). This

could be due to the variation in the nutritional value of the plant tissue consumed (Cockfield, 1988, Mattson, 1980), or because of the variability of certain plant tissue types when the plant experiences stress (for example. changes in xylem sap may be quite different to changes in photosynthetic tissue) (Larsson, 1989, Huberty and Denno, 2004). Alternatively, invertebrate herbivores from different feeding guilds may be differentially exposed to a variety of defensive secondary metabolites (Chen and Andreasson, 2001, Vetter, 2000, Hartmann *et al.*, 1989, Hartmann, 1999, Dafoe and Constabel, 2009), or elicit different induced defence responses (Mewis *et al.*, 2006). Therefore, this study also aims to investigate whether the impact of host identity on the performance of invertebrates feeding on *R. minor* are consistent between phytophagous feeding guilds. In order to achieve this, three generalist invertebrate herbivore species from three distinct feeding guilds were used: a phloem-feeding aphid, *Aphis gossypii,* Glover (Homoptera: Aphididae); a mollusc, *Cornu aspersum,* Müller (Gastropoda: Pulmonata) feeding on leaf tissue; and a xylem-feeding froghopper, *Philaenus spumarius,* Linnaeus (Homoptera: Cercopidae).

In summary, this study aims to answer the following key questions: What is the effect of host plants identity on the growth and performance of *R. minor*? Does the identity of the host plant differentially affect the performance of the hemiparasite's invertebrate herbivores? How do the effects of host identity vary between herbivores feeding on different *R. minor* tissue?

The hypothesis being tested in each case was therefore: (i) Host plant identity will strongly affect the performance of the hemiparasite and will depend on the host's ability to defend its xylem stream. (ii) Invertebrates feeding on the hemiparasite will

be strongly affected by the identity of the host plant, either because of secondary metabolites obtained from their host or because of the relative amounts of nitrogen the hemiparasite is able to remove. (iii) Invertebrate herbivores, from different feeding guilds, feeding on the hemiparasite will be differentially affected by the identity of the host plant because of variation in the nutritional quality of the tissue that they consume.

3.3 Materials and Methods

3.3.1 Plant Material

Rhinanthus minor seed was collected from Castle Hill National Nature Reserve in East Sussex (Grid ref: TQ 375 065) in late July and dried at ambient room temperature. Host plant species were selected on the basis of their presence at the hemiparasite seed source and on the prior work of Gibson and Watkinson (1989) and Cameron *et al*. (2006). These fall into three clear functional groups: legumes (*Lotus corniculatus, Ononis repens* and *Trifolium pratense*); non-leguminous forbs (*Achillea millefolium, Plantago lanceolata* and *Sanguisorba minor*) and grasses (*Briza media, Dactylis glomerata* and *Holcus lanatus*). Host plant seeds (purchased from Emorsgate Seeds, Norfolk) were stored in sealed containers with silica gel at 4°C until required.

R. minor seeds were surface sterilised using 5% household bleach for 60 seconds and then washed four times in sterile water. Approximately 90 seeds were then placed onto 9cm petri dishes containing a damp layer of sterile Whatman glass fibre filter

paper and capillary matting. Petri dishes were then sealed with parafilm and placed in a refrigerated room at 4°C for 84 days.

All plant material was grown in greenhouses at the University of Sussex, in which the temperature was maintained at 15-25°C with supplementary lighting (400w, high pressure sodium lamps) on a 16:8 light:dark regime. Plant and supplied with tap water *ad libitum*. Host plants were germinated on damp vermiculite 28 days after *R. minor* seed stratification had begun. After a further 14 days host plant seedlings were transplanted into 9 cm pots (one per pot) containing six parts sand to one part John Innes No. 2 potting compost. A further set of pots was filled with the growing medium for replicates in which the hemiparasite was grown without a host. 42 days later, five germinating *R. minor* seedlings were transplanted into each pot as required. Hemiparasite seedlings were thinned to one per pot when the majority grown with preferred host plant species (Cameron *et al.*, 2006, Gibson and Watkinson, 1989) showed signs of attachment (Klaren and Janssen, 1978); this was approximately 21 days after *R. minor* seedlings were transplanted.

3.3.2 Interaction Between Host and Hemiparasite

In order to assess the effect of host identity on *R. minor*, and the impact of the hemiparasite on the host's biomass, 25 blocks were set up each containing ten treatments (one of each of the nine host plant species, and one no-host treatment). *R. minor* seedlings were introduced to all treatments in 15 randomly allocated blocks, while the remaining 10 blocks were kept hemiparasite-free.

R. minor height was measured weekly from when the majority of plants had produced their first true leaves (any measurements taken before the hemiparasites were thinned were from the largest plant). The reproductive structures (flower buds,

flowers and seed capsules) of the hemiparasites were counted when the first plants began to show signs of senescence (approximately 15 weeks after their introduction). All the above ground plant material was then harvested, dried at 60°C for 2 days and weighed. Roots were washed and the number of haustoria counted using a binocular dissecting microscope. Final numbers of replicate host plants grown with *R. minor* (and without *R.minor* in brackets) were: *Lotus corniculatus*, n=15 (n=9); *Ononis repens*, n=15 (n=10); *Trifolium pratense*, n=15 (n=10); *Achillea millefolium*, n=12 (n=9); *Plantago lanceolata*, n=7 (n=10); *Sanguisorba minor*, n=15 (n=10); *Briza media*, n=12 (n=10); *Dactylis glomerata*, n=15 (n=10); *Holcus lanatus*, n=15 (n=10) and 'no host', n=15.

3.3.3 Phloem Feeder (*Aphis gossypii*)

The population growth of a generalist aphid (*Aphis gossypii*) was used to evaluate the importance of host identity to invertebrates feeding on the phloem of *R. minor*. Cultures of *Aphis gossypii* were reared on *R. minor* plants grown in trays containing two potential hosts, *Trifolium pratense* and *Holcus lanatus*. As both of these species were also used as hosts in the experiment it should be noted that aphids had had some prior indirect exposure to these species. Nine host plant species were used, plus the 'no host' treatment. Plants were arranged into 20 randomised blocks containing one of each treatment (a total of 200 pots). 70 days after the germinating *R. minor* seeds were introduced, 20 aphids were caged onto the surviving hemiparasites. The final replicate numbers were as follows: *Lotus corniculatus*, n=16; *Ononis repens*, n=13; *Trifolium pratense*, n=13; *Achillea millefolium*, n=16; *Plantago lanceolata*, n=18; *Sanguisorba minor*, n=20; *Briza media*, n=17; *Dactylis glomerata*, n=16; *Holcus lanatus*, n=17 and 'no host', n=17. The aphids were then left to multiply for

14 days, after which they were counted and the above ground portions of both the host and the hemiparasite were harvested, dried at 60°C for 2 days and weighed.

The nitrogen to carbon (N:C) ratios of the above ground portion of the hemiparasites was determined in order to assess if the nitrogen concentration of the hemiparasite plant material was affected by host identity, and whether this in turn affected aphid population growth. This was determined by flash combustion and chromatographic separation of approximately 1.5mg of ground and homogenised plant material, calibrated against a standard compound ($C_{26}H_{26}N_2O_2S$) using an elemental combustion system (Costech Instruments, Milan, Italy).

3.3.4 Xylem Feeder (*Philaenus spumarius*)

The effect of host identity on invertebrates feeding on the xylem stream of the hemiparasite *R. minor* was investigated by comparing the growth rates of spittle bug nymphs (*Philaenus spumarius*). Four of the original nine host plants were selected: *Lotus corniculatus* (n=28), *Ononis repens* (n=26), *Sanguisorba minor* (n= 25) and *Holcus lanatus* (n=25). Plants were arranged into 30 blocks each containing four treatments; all plant material was grown as detailed above.

Second instar *P. spumarius* nymphs feeding on *Lonicera pileata* were collected from Lewes, East Sussex (Grid ref: TQ418099). Nymphs were then weighed and caged onto 70 day old *R. minor* plants. At 48-hour intervals the nymphs were removed, weighed and returned to the hemiparasite. After eight days of feeding on the hemiparasites the relative growth rate (RGR) of the spittle bug ($mg.day^{-1}$) was determined. At this stage the above ground portion of the plant material was harvested, dried at 60°C for two days and then weighed.

3.3.5 Leaf Feeders (*Cornu aspersum*)

Garden snails (*Cornu aspersum*) were used to investigate the effect of host identity on generalist leaf feeders on *R. minor*. Juvenile *C. aspersum* were reared from eggs laid by captive bred stock; all snail cultures (including juveniles) were prior-fed on a diet of lettuce and cucumber. Four host plant species were used and plants were arranged into 20 blocks each containing four treatments, (80 pots in total); all plant material was grown as detailed above. The final replicate numbers were as follows; *Lotus corniculatus*, n=8; *Ononis repens*, n=8; *Dactylis* glomerata, n=10; and *Holcus lanatus*, n=12. Before allowing the juvenile snails to feed on *R. minor* the height of the hemiparasites was measured. Snails of approximately the same size (determined by weight, 0.024g ± 0.004g) were randomly selected, weighed and caged onto the 70-day old hemiparasites. The snails were reweighed every seven days for a total of 21 days in order to determine their RGR.

3.3.6 Cyanogenic Glycoside Assay

The legume *Lotus corniculatus* is known to be polymorphic for the presence of cyanogenic glycosides (Dawson, 1941), which can be an important deterrent to herbivores (Jones, 1962). To determine if the *Lotus corniculatus* plants used were cyanogenic, and whether these cyanogens were passed to the hemiparasite *R. minor*, I used a standard picrate paper assay (Egan *et al.*, 1998, Bradbury *et al.*, 1999).

Host plants and parasites were grown as described previously; two host plants were used, *Lotus corniculatus* (thought to be cyanogenic) and *Ononis repens* (assumed to be acyanogenic). Seeds were selected from the same source as all the previous experiments. 100mg of fresh plant material was collected from *R. minor* and the host plant to which it was attached (70 days after the hemiparasite was introduced). This

was then ground using a pestle and mortar, and placed in an air tight container along with 5ml of 0.1M phosphate buffer solution and the pre-prepared picrate paper (filter papers dipped into a solution of picric acid (0.5% w/v) in 2.5% (w/v) sodium carbonate and allowed to dry). The containers were then stored at room temperature and in the dark for 24 hours. The presence of cyanogenic glycosides was then determined by a change in colouration of the picrate paper from yellow to orange.

3.4 Results

3.4.1 Interaction Between Host and Hemiparasite

The presence of the hemiparasite significantly reduced the final above ground biomass of the host plants *Holcus lanatus* and *Sanguisorba minor* (t-test, $t_{20} = 2.39$, $P = 0.025$ and $t_{15} = 3.45$, $P = 0.002$ respectively). No other host species had their biomass significantly decreased by being parasitized, and some (*Dactylis glomerata*, *Ononis repens* and *Trifolium pratense*) showed a slight, although non-significant, increase in biomass (Figure 1).

The identity of the host had a highly significant effect on the height and the final biomass of the hemiparasite (height $F_{9,120} = 13.93$, $P < 0.001$; biomass $F_{9,120} = 18.71$, $P < 0.001$). With the exception of *Trifolium pratense,* the hemiparasite attained the greatest biomass when attached to the leguminous hosts, and least when attached to *Plantago lanceolata* and when grown without a host (Figure 2).

The number of haustorial connections between the host and the hemiparasites were strongly affected by the identity of the host (Kruskal-Wallis, $H_8 = 51.88$, $P < 0.001$), and there was a highly significant correlation between the hemiparasite's final biomass and the number of haustoria (Spearman rank-order $r_{2,\ 104} = 0.685$, $P <$

0.001). Furthermore, for the five host plant treatments which had the highest mean hemiparasite biomass, there was a significant positive correlation between the number of haustoria and the final biomass of *R. minor* plants (Table 1), but for the four host plant treatments with the lowest mean *R. minor* biomass there was no significant correlation.

Host identity had a strong effect on the number of reproductive structures produced (Krukal-Wallis, $H_8 = 71.42$, $P < 0.001$) with plants attached to *L. corniculatus* and *O. repens* producing the most, and grown with *P. lanceolata, A.millifolium* and the no-host treatment producing the least (mean number of flowering structures 19.9, 25.0, 0.1, 1.5 and 0.9 respectively). Finally, the biomass of the hemiparasite and the number of reproductive structures produced were highly correlated (Spearman rank-order $r_{2,\ 113} = 0.870$, $P < 0.001$).

3.4.2 Phloem Feeder (*Aphis gossypii*)

With the exception of hemiparasites growing with the host plant *B. media*, the final biomass of the hemiparasite followed a broadly similar pattern to that of the previous experiment with host identity significantly affecting both final height and the final biomass of the hemiparasite (Kruskal-Wallis, height, $H_8 = 65.16$, $P < 0.001$, biomass $H_8 = 76.09$, $P < 0.001$). The increased growth of *R. minor* plants growing with *B. media* might be explained by early senescence of the host, potentially leading to an increase in the available nitrogen to the hemiparasite (White, 1984, Mattson, 1980).

The performance of the aphids was strongly affected by the identity of the host ($F_{9,157} = 11.78$, $P < 0.001$), with an average increase of over 230 individuals over 14 days for aphids feeding on *R.minor* plants attached to *O. repens* compared to an increase of less than 30 individuals on hemiparasites attached to *L. corniculatus*

(Figure 3). The number of aphids per mg of *R. minor* was also strongly affected by the identity of the host (Kruskal-Wallis: H_9 = 69.82, P < 0.001). Hemiparasites grown with *P. lanceolata* and those grown without a host (the two treatments with the lowest hemiparasite biomass) having the most aphids per mg of *R. minor*, while those grown with *L. corniculatus* had the least number of aphids per mg after 14 days (Figure 4).

The mean nitrogen to carbon ratios (N:C) of the hemiparasites were strongly affected by host plant identity (Kruskal-Wallis: H_9 = 72.18 P < 0.001). Unsurprisingly, the highest N:C ratios were found when *R. minor* was attached to leguminous hosts (Figure 5). Correlations between the mean number of aphids after 14 days and the mean N:C ratio (Figure 6) and the hemiparasite biomass, were not significant (Pearsons product moment correlation, N:C, $r_{2,\ 8}$ = 0.174, P = 0.63, biomass $r_{2,\ 8}$ = 0.333, P = 0.347). Removal of the *L. corniculatus* treatment gave significant correlations for both (Pearsons product moment correlation, N:C, $r_{2,\ 7}$ = 0.746, P = 0.021, biomass, $r_{2,\ 7}$= 0.700, P = 0.036).

3.4.3 Xylem Feeder (*Philaenus spumarius*)

With the exception of hemiparasites growing with the host *O. repens*, which had an unusually low final biomass, the performance of the hemiparasites was broadly comparable to the previous experiments, with significant differences in the final biomass across the four host plant treatments (Kruskal-Wallis: H_3 = 35.23, P < 0.001).

Host identity also had a large and significant effect on the relative growth rate (RGR) of *P. spumarius* ($F_{3,100}$ = 7.12, P < 0.001), with froghoppers feeding on hemiparasites attached to *O. repens* consistently having mean RGRs below those of

the other three host treatments (*L. corniculatus, S. minor* and *H. lanatus*) (Figure 7), which may simply reflect the unusually low *R.minor* biomass on this particular host. *Post-hoc* analysis reveals that the remaining three treatments show no significant differences in RGR ($P = 0.05$). This raises the possibility that the performance of this xylem-feeding generalist is affected more by the performance of the hemiparasite than by the identity of its host. There was a strong positive correlation between the RGR of *P. spumarius* and the biomass of the hemiparasite across all treatments (Spearman rank-order, $r_{2,102} = 0.342$, $P < 0.001$), suggesting that this could indeed be the case.

3.4.4 Leaf Feeder (*Cornu aspersum*)

The final heights of the hemiparasites were not significantly affected by host identity (Kruskal-Wallis: $H_3 = 2.54$ $P = 0.468$). Host identity did, however, have a significant effect on the RGR of the juvenile snails after 7, 14 and 21 days, with snails feeding on *R. minor* grown with *O. repens* consistently having the highest RGR and those feeding on *R. minor* attached to *L. corniculatus* consistently having the lowest RGR (Table 2 a). After 21 days the snails feeding on the hemiparasites attached to *O. repens* had significantly larger RGRs than those feeding on hemiparasites attached to the other three hosts (*L.corniculatus, D. glomerata* and *H. lanatus*), with no significant differences between the other three treatments (ANCOVA main factor, $F_{3,33} = 4.70$ P = 0.008, covariate (plant height) $F_{1,33} = 42.90$, $P < 0.001$ and Tukey *post-hoc* analysis $P = 0.05$).

3.4.5 Cyanogenic Glycoside Assay

Colour changes in the picrate papers confirmed that *L. corniculatus* plants used in my experiments were cyanogenic, but no cyanogenic glycosides were detected in *R.*

minor attached to this host. As expected, neither *Ononis repens* nor the hemiparasite attached to it showed any sign of cyanogenesis.

3.5 Discussion

Previous experiments have clearly shown that the identity of the host can have strong effects on the performance of *R. minor* (Seel *et al.*, 1993a, Cameron *et al.*, 2006), and that this reflects both the host plant's ability to defend itself from the hemiparasite (Rumer *et al.*, 2007, Cameron and Seel, 2007, Cameron *et al.*, 2006) and the amount of nitrogen available in the host plant (Seel *et al.*, 1993a). This study supports these findings, with hemiparasites performing well when grown with nitrogen rich legumes (with the exception of *Trifolium repens*). The lack of a relationship between haustorial connections and biomass for the worst performing hemiparasites suggests that some host plants were successfully defending their xylem stream against attachment by *R. minor* (e.g. *Plantago lanceolata*). In addition, this study has also shown that the identity of the host plants indirectly affects the performance of *R. minor's* invertebrate herbivores. The patterns in the performance of different guilds of invertebrate herbivores feeding on hemiparasites are not however, consistent, with the performance of xylem-feeding spittle bug being quite different to that of phloem feeding-aphids and tissue-rasping snails.

For the generalist aphid, *Aphis gossypii*, feeding on *R. minor*, it appears that the hemiparasite's biomass and the relative amounts of nitrogen in its tissue are, in most cases, the most important factors affecting their reproductive rate. This is perhaps not surprising as previous work on this aphid species has shown that increases in the nitrogen content of the leaves of cotton plants lead to a marked increase in its

performance (Nevo and Coll, 2001). The reproductive rate of aphids feeding on the hemiparasites attached to *Lotus corniculatus* were, however, lower than for any other treatment, despite the hemiparasites on this host having the highest mean biomass and nitrogen to carbon ratios. The performance of the snails was similar to that of the aphids in that snails feeding on *L. corniculatus* had lower relative growth rates than snails feeding on hemiparasites attached to the other leguminous host.

One possible explanation for the decreased performance observed in snails and aphids feeding on *R. minor* plants grown with *L. corniculatus* is the presence of defensive compounds in hemiparasites attached to this host. The increased rates of photosynthesis that have been associated with hemiparasites attaching to nitrogen rich leguminous hosts (Seel *et al.*, 1993a, Press, 1995) could lead to an increase in the hemiparasite's own defensive secondary metabolites. This, however, would seem an unlikely explanation, as both the snails and the aphids performed best on hemiparasites attached to *O. repens*, another legume. A more plausible explanation is that a defensive compound was passed from the host to the hemiparasites. This has been shown for several species of parasitic plant (*Castilleja spp* (Adler and Wink, 2001, Marvier, 1996), *Cuscuta palaestina* (Wink and Witte, 1993), *Euphrasia stricta* (Rasmussen *et al.*, 2006), and *Pedicularis semibarbata* (Stermitz *et al.*, 1989)) including *Rhinanthus serotinus* (Lehtonen *et al.*, 2005). In this latter example, a defensive alkaloid was passed from the host plant to the hemiparasite and reduced the performance of aphids feeding on the hemiparasite. *Lotus corniculatus* is known to produce a variety of secondary metabolites (Jones and Turkington, 1986), the most notable of which is a cyanogenic glycoside that has been shown to deter herbivores (Jones, 1962). No cyanogenic glycosides were however, detected in the tissue of *R. minor* plants attached to cyanogenic *L. corniculatus* plants. While *L.*

corniculatus is also known to contain high levels of condensed tannins (Briggs and Schultz, 1990), another group of secondary metabolites considered to play an important role in plant defence (Bennett and Wallsgrove, 1994), the size and chemical structure of these molecules mean they are unlikely candidates for the effects observed here (Zucker, 1983). Other species within the *Lotus* genus have been shown to contain various nitro-bearing molecules which can also be toxic to invertebrates (Williams, 1983, Gnanasunderam and Sutherland, 1986), but their presence in *L. corniculatus* is yet to be confirmed. It appears therefore, that further work will be required in order to identify the compounds potentially responsible for this effect.

In complete contrast to the other two phytophagous species, *Philaenus spumarius* had highest relative growth rates when feeding on *R. minor* plants attached to *L. corniculatus*. *P. spumarius*, however, is known to feed on a very wide variety of plants including *L. corniculatus* (Weaver and King, 1954), so it is perhaps unsurprising that indirect contact with this plant produced no negative effects on the froghopper. Previous studies have shown that *P. spumarius* preferentially feeds on legumes (Thompson, 1994) and plants that are richer in amino acids (Horsfield, 1977). Thus the performance of *P. spumarius* in this study appears to be related primarily to the performance of the hemiparasite rather than to either the identity or the performance of its hosts.

This study suggests that forming haustorial attachments with some leguminous host plants presents a considerable advantage to *R. minor*. Hemiparasite-induced changes in the plant community (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Bullock and Pywell, 2005, Bullock *et al.*, 2007, Davies *et al.*, 1997, Gibson and Watkinson, 1992,

Ameloot *et al.*, 2005) and the highly dynamic population cycles of *R. minor* (Cameron *et al.*, 2009) may however mean that it is not possible to form attachments regularly with any given host species. Consequently, for herbivores, *R. minor* may represent a food resource whose quality is continuously changing depending on its pattern of host attachment. As suggested by Lehtonen *et al* (2005), this could well explain why plants of this genus have relatively few specialist herbivores (only four are recorded for *R. minor* on the Phytophagous Insect Database). Furthermore, the ability of certain host plants to provide the hemiparasite with a degree of defence against herbivores, coupled with the variation in nitrogen provided by some host plants means that *R. minor* is a highly variable food source. The ability of the parasites to form haustorial connections with numerous host plants simultaneously (Gibson and Watkinson, 1989) provides additional complexity for herbivores.

Despite the complex nature of the host-hemiparasite-invertebrate interaction, this study has shown that for invertebrates using *R. minor* as a food source, the identity of the host to which the hemiparasite attaches is of utmost importance for herbivore performance. Furthermore, the impact of the hemiparasite on its host observed here and in other studies (Cameron *et al.*, 2008, Seel and Press, 1996), and their impact on plant communities (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Bullock and Pywell, 2005, Bullock *et al.*, 2007, Davies *et al.*, 1997, Gibson and Watkinson, 1992, Ameloot *et al.*, 2005) may have important implications for invertebrate herbivores, affecting both individual species and potentially whole communities.

3.6 Tables

Table 1. The relationship between the final biomass of *R. minor* and the number of haustorial connections after the hemiparasite had been grown with the host plant for 110 days.

Host identity	**n**	***R. minor* Biomass (g) = y**		**Haustorial Connections = x**		**Spearman rank-order correlation (x *vs.* y)**		**Straight line regression equation**
		Mean	SEM	Mean	SEM	r_2 =	p =	
Briza media	10	0.22	0.06	16.30	2.72	0.228	0.527	y = -0.001x + 0.2072
Dactylis glomerata	15	0.29	0.06	15.47	3.31	0.681	0.005	y = 0.0145x + 0.0693
Holcus lanatus	12	0.61	0.11	29.42	7.70	0.867	<0.001	y = 0.012x + 0.2537
Achillea millefolium	11	0.29	0.08	17.18	4.47	0.312	0.350	y = -0.00002x + 0.2883
Plantago lanceolata	7	0.07	0.01	14.00	5.76	0.667	0.102	y = 0.0015x + 0.046
Sanguisorba minor	15	0.57	0.07	56.60	7.89	0.670	0.006	y = 0.0055x + 0.2541
Lotus corniculatus	15	1.17	0.19	43.00	8.42	0.571	0.026	y = 0.0146x + 0.5432
Ononis repens	15	1.15	0.30	95.60	20.88	0.543	0.037	y = 0.0127x - 0.0664
Trifolium pratense	6	0.25	0.10	11.00	2.77	0.771	0.072	y = 0.0302x - 0.0774
Grand Mean	**106**	**0.59**	**0.07**	**38.01**	**4.40**	**0.685**	**<0.001**	**y = 0.0116x + 0.149**

Table 2 a, b. (a) The mean relative growth rate (RGR), and (b) the statistical analysis (ANCOVA) of juvenile snails feeding on *Rhinanthus minor* plants that were grown with various host plants. Hemiparasite height, as a proxy for hemiparasite biomass, was used as the covariate. Host plants: *Dactylis glomerata* (DG), *Holcus lanatus* (HL), *Lotus corniculatus* (LC) and *Ononis repens* (OR).

Host Identity	**Mean RGR mg/day (± SEM)**		
	0-7 days	**0-14 days**	**0-21 days**
DG	0.556 (± 0.343)	0.781 (± 0.415)	0.833 (± 0.449)
HL	0.506 (± 0.364)	0.571 (± 0.255)	0.578 (± 0.367)
LC	0.129 (± 0.228)	0.201 (± 0.200)	0.176 (± 0.234)
OR	1.770 (± 0.551)	1.662 (± 0.359)	1.801 (± 0.402)

Factor	**Analysis of Covariance**		
	0-7 days	**0-14 days**	**0-21 days**
Main factor	P=0.040	P=0.020	P=0.008
	$F_{3,36}$=3.1	$F_{3,34}$=3.7	$F_{3,33}$=4.7
Covariate	P=0.003	P<0.001	P<0.001
	$F_{1,36}$=10.6	$F_{1,34}$=21.3	$F_{3,33}$=42.9

3.7 Figures

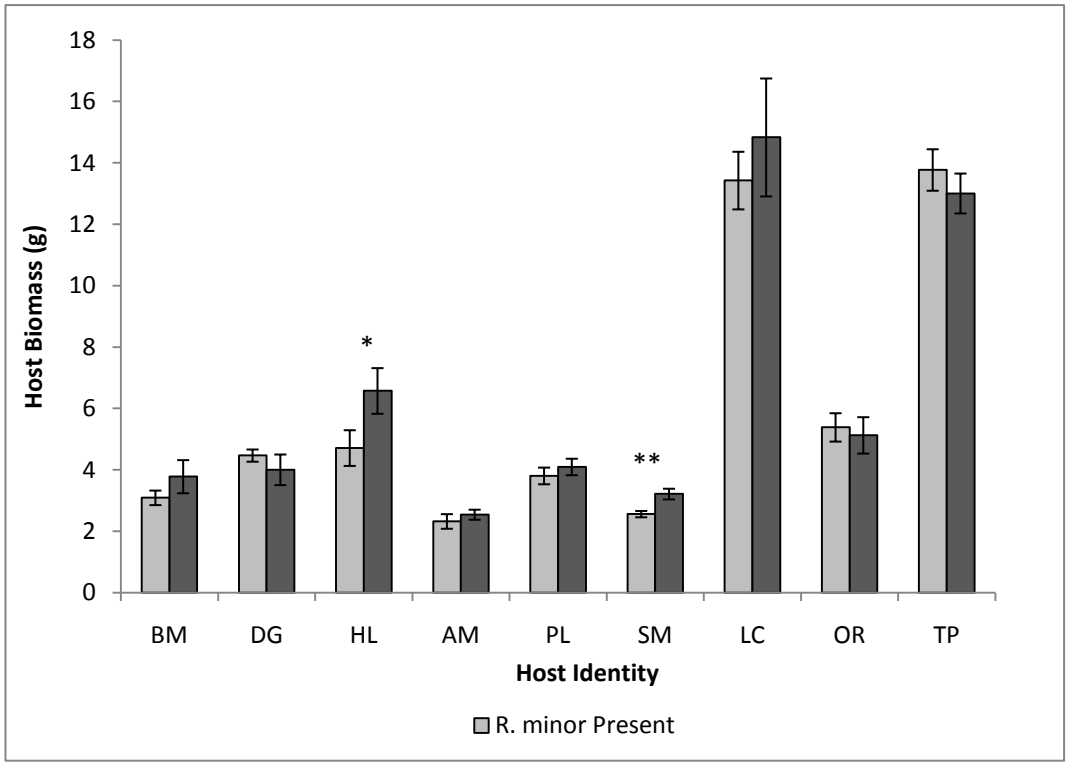

Figure 1. The impact of *R. minor* on its host's biomass. Error bars show ± 1 SEM, Two-sample t-test: t_{20} = 2.39, P = 0.025*, t_{15} = 3.45 P = 0.002**, all other pairs were not statistically different. Where necessary, the data were log10 or square root transformed to meet the assumptions of the test statistic. Grasses: *Briza media* (BM), *Dactylis glomerata* (DG) and *Holcus lanatus* (HL). Non-leguminous forbs: *Achillea millefolium* (AM), *Plantago lanceolata* (PL) and *Sanguisorba minor* (SM). Legumes: *Lotus corniculatus* (LC), *Ononis repens* (OR) and *Trifolium pratense* (TP).

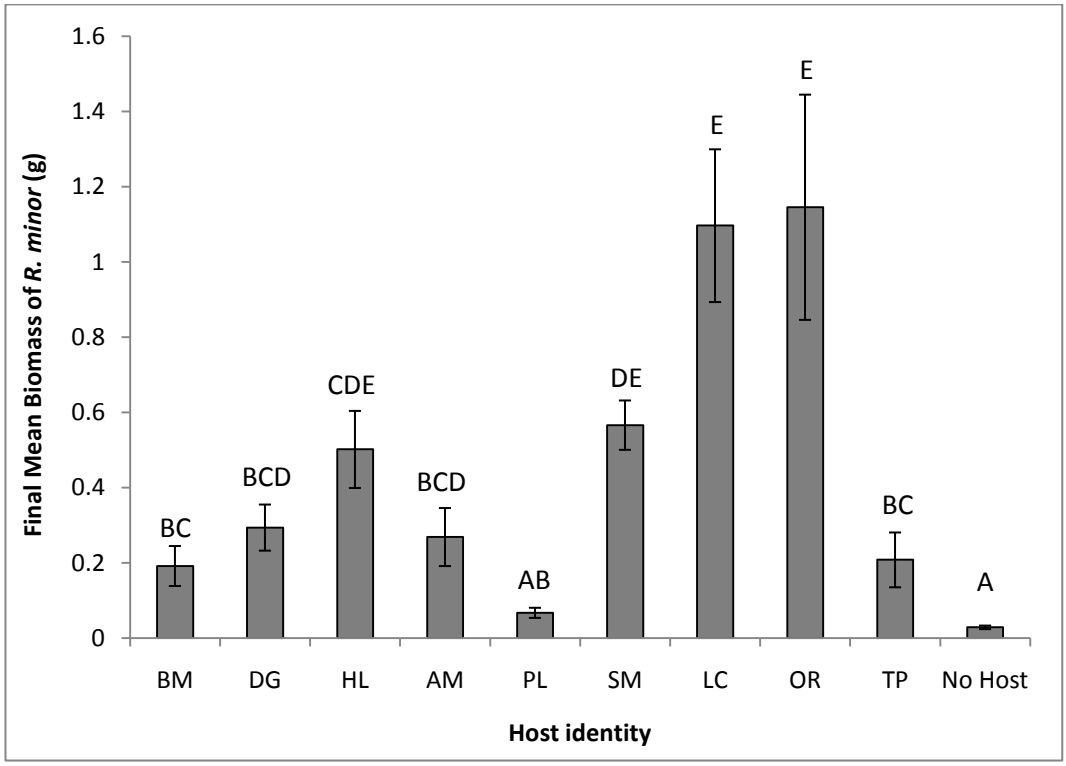

Figure 2. Mean biomass in grams (g) achieved by *Rhinanthus minor* when grown with various host species for 15 weeks. Error bars show ± 1 SEM. One-way ANOVA: $F_{9,120}$ = 18.71, P < 0.001. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis P = 0.05). Values were log10 transformed to meet the assumptions of the test statistic. Grasses: *Briza media* (BM), *Dactylis glomerata* (DG) and *Holcus lanatus* (HL). Non-leguminous forbs: *Achillea millefolium* (AM), *Plantago lanceolata* (PL) and *Sanguisorba minor* (SM). Legumes: *Lotus corniculatus* (LC), *Ononis repens* (OR) and *Trifolium pratense* (TP).

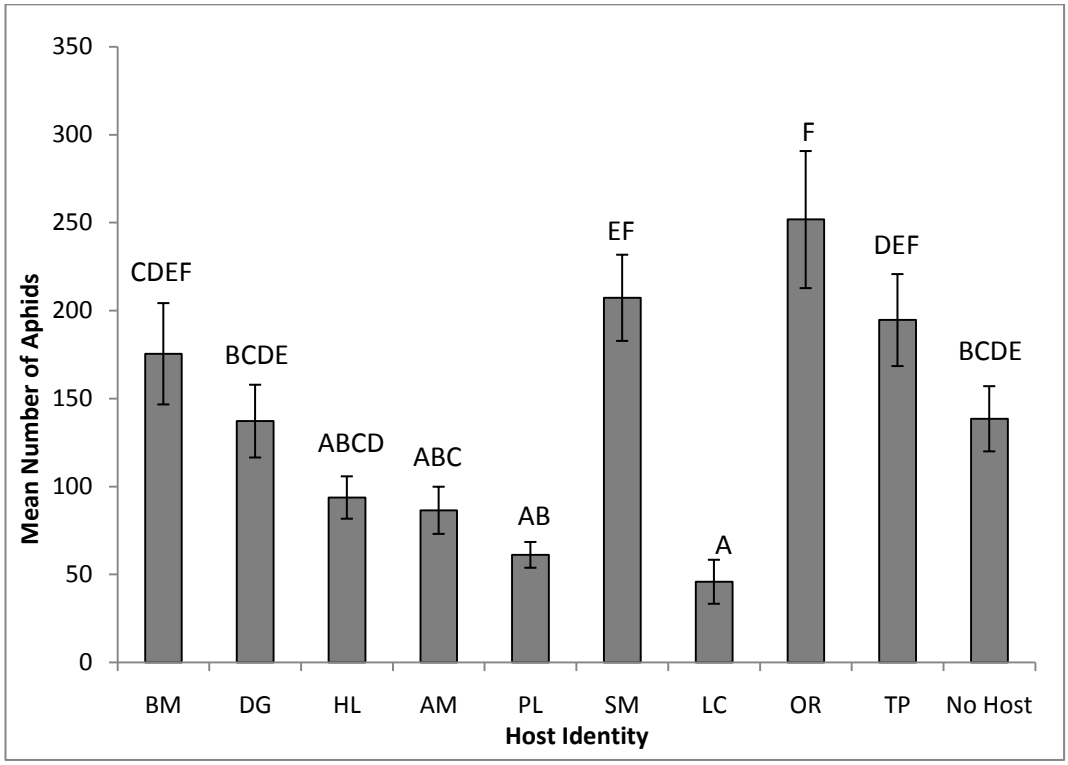

Figure 3. The mean number of Aphids after 14 days on *R. minor* plants grown with various host plants (starting density = 20 aphids). Error bars show ± 1SEM. One-way ANOVA $F_{9,157} = 11.78$ $P < 0.001$. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis $P=0.05$). Data were transformed using the Freeman Tukey transformation equation $(((\sqrt{\chi}) + (\sqrt{\chi + 1})) / 2)$ to meet the assumptions of the test statistic. Grasses: *Briza media* (BM), *Dactylis glomerata* (DG) and *Holcus lanatus* (HL). Non-leguminous forbs: *Achillea millefolium* (AM), *Plantago lanceolata* (PL) and *Sanguisorba minor* (SM). Legumes: *Lotus corniculatus* (LC), *Ononis repens* (OR) and *Trifolium pratense* (TP).

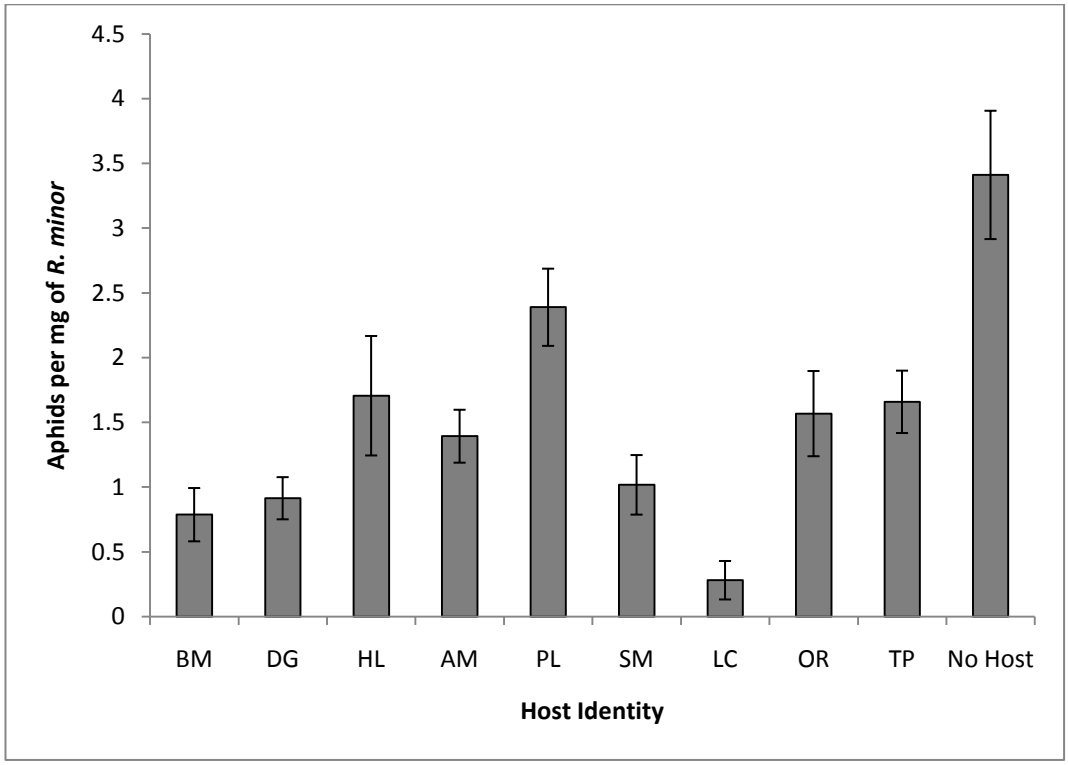

Figure 4. The mean number of Aphids per mg of *R. minor* after 14 days on *R. minor* plants grown with various host plants (starting density = 20 aphids). Error bars show ± 1SEM. Kruskal-Wallis: H_9 = 69.82, P < 0.001. Grasses: *Briza media* (BM), *Dactylis glomerata* (DG) and *Holcus lanatus* (HL). Non-leguminous forbs: *Achillea millefolium* (AM), *Plantago lanceolata* (PL) and *Sanguisorba minor* (SM). Legumes: *Lotus corniculatus* (LC), *Ononis repens* (OR) and *Trifolium pratense* (TP).

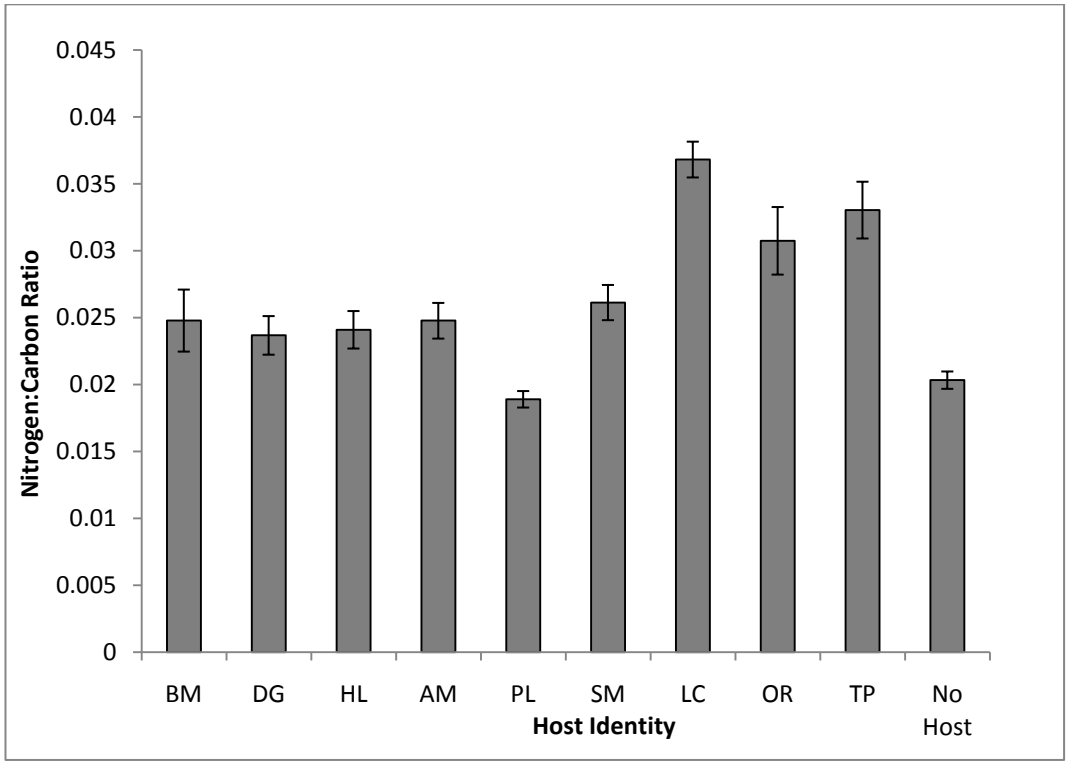

Figure 5. Mean Nitrogen to Carbon ratio of *Rhinanthus minor* plants when grown with various host species. Error bars show ±1SEM of the mean. Kruskal-Wallis: $H_9 = 72.18$, $P < 0.001$ Grasses: *Briza media* (BM), *Dactylis glomerata* (DG) and *Holcus lanatus* (HL). Non-leguminous forbs: *Achillea millefolium* (AM), *Plantago lanceolata* (PL) and *Sanguisorba minor* (SM). Legumes: *Lotus corniculatus* (LC), *Ononis repens* (OR) and *Trifolium pratense* (TP)

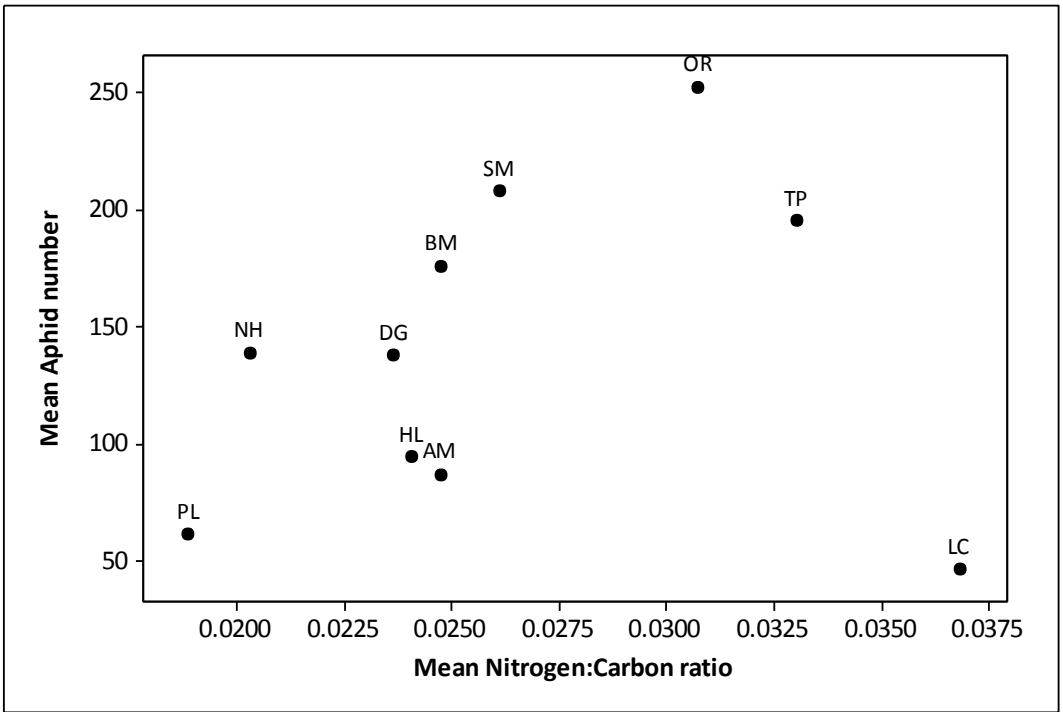

Figure 6.The mean Nitrogen to Carbon ratio of *Rhinanthus minor* plants against the mean number of aphids after 14 days on those plants (starting density = 20), points represent means for each host plant treatment. Pearson product-moment correlation: $r_{2,8}$ = 0.174, *P* = 0.63 (regression line: y = 2098.6x + 84.013). With LC removed: Pearson product moment correlation $r_{2,7}$ = 0.746, *P* = 0.021 (regression line: y = 10483x - 114.18). Grasses: *Briza media* (BM), *Dactylis glomerata* (DG) and *Holcus lanatus* (HL). Non-leguminous forbs: *Achillea millefolium* (AM), *Plantago lanceolata* (PL) and *Saguisorba minor* (SM). Legumes: *Lotus corniculatus* (LC), *Ononis repens* (OR) and *Trifolium pratense* (TP). No Host (NH).

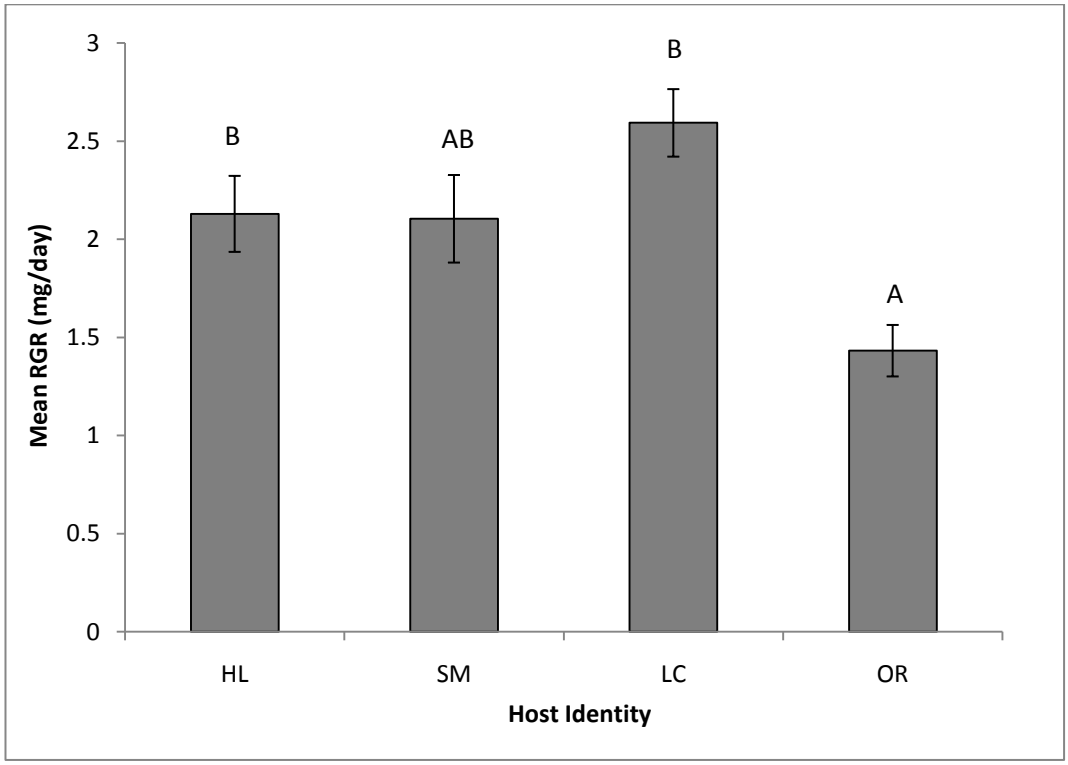

Figure 7. The mean relative growth rate (RGR) after 8 days of *Philaenus spumarius* nymphs feeding on *Rhinanthus minor* plants that were grown with various host plants. Error bars show ± 1 SEM. One-way ANOVA $F_{3,100} = 7.12$, $P < 0.001$. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis $P=0.05$). Host plants: *Holcus lanatus* (HL), *Sanguisorba minor* (SM), *Lotus corniculatus* (LC) and *Ononis repens* (OR).

Chapter 4. The Effect of Multiple Hosts on the Performance of the Hemiparasite *Rhinanthus minor* and its Invertebrate Herbivores

4.1 Abstract

The hemiparasite *Rhinanthus minor* is a common component of many grassland communities and is capable of attaching to a very broad range of host plants. The identity of these host species can have a major impact on the performance of the hemiparasite and on that of the invertebrate herbivores that feed on it. In many cases the host may influence the performance of the invertebrate herbivores of *R. minor* via its impact on the hemiparasite's nutrient quality. *R. minor* can potentially acquire defensive compounds as well as nutrients from its host which might greatly reduce the performance of the hemiparasite's invertebrate herbivores. In addition, under natural conditions *R. minor* can form attachments with multiple host plants, some that may transfer defensive compounds and some that do not. This means that the majority of host-hemiparasite-herbivore interactions are likely to be moderated by the presence of other hosts, particularly if they are of high quality for the hemiparasite.

The aims of the current study were to investigate (i) how the performance of the hemiparasite *R. minor* was affected by the presence of more than one host species and (ii) whether the impacts of a host plant that adversely effect the herbivores of *R minor* are mitigated by the presence of additional host species.

The results demonstrate that for the majority of host plant species combinations, having more than one host species is a genuine advantage to *R. minor*, and hemiparasites grown with mixed hosts generally attain greater biomass. This advantage, however, does not seem to be apparent when the hemiparasites are grown with two closely related host plants. Importantly, the invertebrate herbivore resistance conferred by the host plant *L.corniculatus* is still apparent, if somewhat reduced, in the presence of a second host plant species. An investigation of the haustorial connections between the *R. minor* and its hosts suggests the level of resistance conferred by *L.corniculatus* are related to the proportion of attachments made to this plant. Overall, this study demonstrates the importance of the hosts' identity to the performance of *R. minor* and its invertebrate herbivores. Furthermore, the study shows that the patterns in parasitic attachment can have major implications for this hemiparasite's ecological interactions.

Key words: Hemiparasite; *Rhinanthus minor*; host parasite interaction; invertebrate herbivores; indirect interaction.

4.2 Introductions

Parasitic plants can have major and important impacts on ecological communities (Irving and Cameron, 2009, Phoenix and Press, 2005, Press and Phoenix, 2005). Their presence can have dramatic effects on plant community structure (Pennings and Callaway, 1996, Ameloot *et al.*, 2005, Joshi *et al.*, 2000), nutrient cycling (Quested *et al.*, 2003a, Quested *et al.*, 2002, Quested *et al.*, 2003b) and may also indirectly affect the performance of invertebrate herbivores (Puustinen and Mutikainen, 2001).

One parasitic plant species that has received considerable attention over recent years is the hemiparasite *Rhinanthus minor*. This plant is a common component of many temperate grassland ecosystems constituting as much as 8% of standing plant biomass at certain times of the year (Davies *et al.*, 1997). It has also been shown to have major impacts on ecosystem processes (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Bullock and Pywell, 2005, Bullock *et al.*, 2007, Davies *et al.*, 1997, Gibson and Watkinson, 1992, Ameloot *et al.*, 2005, Bardgett *et al.*, 2006, Ameloot *et al.*, 2008). So far most previous studies on this hemiparasite have focused on the interactions between *R. minor* and the plant community (but see Bardgett *et al.*, 2006), and relatively little is known about its interactions with invertebrate herbivores.

Like many other hemiparasitic plants, *R. minor* attaches to the xylem stream of its host plants via specialised organs known as haustoria (Kuijt, 1969, Riopel and Timko, 1995). *R. minor* has been shown to form these haustorial connections with around 50 different plant species from 18 families (Gibson and Watkinson, 1989) and, as with many hemiparasites, its performance varies widely depending on the species to which it is attached (Seel *et al.*, 1993a, Cameron *et al.*, 2006). The variation in the performance of *R. minor* is largely due to the host plant's ability to defend its xylem stream from the hemiparasite (Cameron *et al.*, 2006, Rumer *et al.*, 2007, Cameron and Seel, 2007) and variation in the types and amounts of solutes that the hemiparasite can remove from its host (Seel *et al.*, 1993a, Press, 1995).

This variation in the quality of the host not only affects the performance of *R. minor* but also indirectly affects the performance of the invertebrate herbivores feeding on it (Chapter 3). Nutrient content, and in particular the nitrogen to carbon (N:C) ratio are key factors affecting the performances of invertebrate herbivores feeding on the

hemiparasite. But it is not just nutrients that are acquired by the hemiparasite from the host. For certain host-hemiparasite associations, the hemiparasite may acquire defensive compounds from the host which can greatly reduce the performance of its invertebrate herbivores. Previous work on *R. minor* shows that hemiparasites grown with the legume, *Lotus corniculatus*, acquire some, as yet unidentified compound, which greatly reduce the performance and growth rate of aphids and snails feeding on the hemiparasite (Chapter 3). A potential mechanism is the transfer of defensive secondary metabolites from hosts to hemiparasites, which has been well documented for several other parasitic plant species (Adler and Wink, 2001, Wink and Witte, 1993, Rasmussen *et al.*, 2006, Stermitz *et al.*, 1989, Lehtonen *et al.*, 2005). Furthermore, this phenomenon has been shown to deter invertebrate herbivores (Adler *et al.*, 2001, Adler, 2000, Adler, 2002, Adler, 2003) and decrease their growth and performance (Marvier, 1996).

In common with many other hemiparasites, *R. minor* regularly forms attachments with multiple hosts simultaneously (Gibson and Watkinson, 1989, Pate *et al.*, 1994, Matthies, 1996). This has important implication for both the performance of the hemiparasite and the performance of its invertebrate herbivores because any of the advantages, or indeed disadvantages, of attaching to host plants of a certain species may be moderated by the presence of additional host plants. Indeed, Govier *et al* (1967) suggested that because parasitic plants gain a diversity of different compounds from different host species, attachment to multiple hosts may provide a considerable advantage. In addition to this, hemiparasites attaching to multiple hosts may capitalise on the ability of certain host plants to tolerate environmental stress. For example, Pate *et al* (1990) suggested that attaching to a deep-rooted host plant provided the hemiparasite *Olax phyllanthii* with a considerable advantage during

drought stress. Experimental work on hemiparasites utilising multiple host plants under controlled environments has yielded somewhat mixed results, with some hemiparasite species apparently benefiting from more than one host plant species, while others do not (Marvier 1998; Matthies 1996). Moreover, the evidence of any indirect effects from multiple host species on the invertebrate herbivores of hemiparasitic plants is somewhat limited and confined to a single hemiparasite species, *Castilleja wightii* (Marvier, 1998).

The primary aim of this study was to investigate how the presence of two different host species affects the performance of the hemiparasite *R. minor*, and how this in turn indirectly affects the performance of the hemiparasite's insect herbivores. In addition to this, the study aims to investigate whether the negative impacts of the host plant *Lotus corniculatus* (Chapter 3) on the herbivores of *R. minor* are mitigated by the presence of additional host species.

By growing *R. minor* with a variety of host plants simultaneously I aimed to answer the following questions: Is the performance of the hemiparasite enhanced by the presence of more than one host plant species? Are the defensive properties acquired by *R. minor* from the host plant *L. corniculatus* moderated by the presence of a second host plant species?

The hypothesis being tested in each case was therefore: (i) Having access to more than one host plant species will enhance performance of the hemiparasite because it will have access to a wider variety compounds. (ii) The defensive properties conferred by the host plant *L. corniculatus* will be diluted by the presence of a second host plant species.

4.3 Methods

4.3.1 Experimental Design

Experiments consisted of three treatments: in the first, the hemiparasite had access solely to *L. corniculatus*; in the second, the parasite had access to both *L. corniculatus* and a second host plant species which did not appear to confer strong herbivore resistance to the hemiparasite; finally, in the third, the hemiparasite had access only to this second host plant species. Therefore, each experimental treatment consisted of *Rhinanthus minor* plants grown with either a homospecific pair of host plants or to two different host plant species. The additional host plant species were selected from three separate functional groups on the basis of my previous work (Chapter 3) and on the prior work of Gibson and Watkinson (1989) and Cameron *et al* (2006): *Ononis repens* (legume), *Sanguisorba minor* (non-leguminous forb) and *Dactylis glomerata* (grass), which suggests that they are good hosts for the hemiparasite. Each experiment consisted of 20 blocks each containing one replicate of each of the three treatments (60 pots in total).

4.3.2 Plant Material

Rhinanthus minor seed was collected from Castle Hill National Nature Reserve in East Sussex (Grid ref: TQ 375 065) in late July and dried at ambient room temperature. Host plant seeds (purchased from Emorsgate Seeds, Norfolk) were from the same source and batch as those used in my previous experiments (Chapter 3). All seeds were stored in sealed containers with silica gel at 4°C until required.

Prior to stratification *R. minor* seeds were surface sterilised using 5% household bleach for 60 seconds and then washed four times in sterile water. Seeds were then placed onto 9cm petri dishes (approximately 90 seeds per dish) containing a damp

layer of sterile Whatman glass fibre filter paper and capillary matting. Petri dishes were then sealed with parafilm and placed in a refrigerated room at 4°C for 84 days.

Host plants were germinated on damp vermiculite 28 days after *R. minor* seed stratification had begun. After a further 14 days host plant seedlings were transplanted, in the paired combinations described above, to 9 cm pots containing six parts sand to one part John Innes No. 2 potting compost. 42 days later, five germinating *R. minor* seedlings were transplanted into each pot. Hemiparasite seedlings were thinned to one per pot when the majority showed signs of attachment (Klaren and Janssen, 1978), which was approximately 21 days later.

All experiments were carried out in greenhouses at the University of Sussex. The temperature was maintained at 15-25°C with supplementary lighting (400w, high pressure sodium lamps) on a 16:8 light:dark regime and plants were supplied with tap water *ad libitum*.

4.3.3 Hemiparasite and Aphid Performance

The population growth of a generalist aphid (*Aphis gossypii*) was used to evaluate herbivore performance. Cultures of *A. gossypii* were reared on *R. minor* plants grown in trays containing two potential host plants, *Trifolium pratense* and *Holcus lanatus*, so that they had no prior indirect exposure to the host plants used in this experiment. 70 days after the *R. minor* seedlings were introduced into the pots, 20 aphids were caged onto the surviving hemiparasites. The aphids were then left to multiply for 14 days, at which point all aphids were counted and the above ground portions of both the host and the hemiparasite were harvested, dried at 60°C for 2 days and weighed.

4.3.4 Haustorial Attachments

To examine the relationship between the pattern of haustorial attachment and the performance of the aphids, the below ground portion of the plant material was washed and the number of haustoria and the host plant species to which they were attached (where appropriate) were counted. Given the difficulty in distinguishing visually between roots of different species, it was only possible to assign haustoria reliably to a particular host plant in the experiment involving *O. repens.*

4.3.5 Nitrogen:Carbon Ratio

The nitrogen to carbon (N:C) ratios of the above ground portion of the hemiparasites was determined in order to assess if the nitrogen concentration of the hemiparasite plant material was affected by the experimental host plant combination, and whether patterns in aphid population growth were affected by the N:C ratios. Eight replicates from each of the experimental treatments were randomly selected. The N:C ratios of the parasites were determined by flash combustion and chromatographic separation of approximately 1.5mg of ground and homogenised plant material, using an elemental combustion system (Costech Instruments, Milan, Italy) and calibrated against a standard compound ($C_{26}H_{26}N_2O_2S$) (all treatments n=8).

4.3.6 Statistical Analysis

All statistical analyses were done using Minitab 15 for Windows (Minitab, Inc). Whenever possible the effect of experimental block was analysed with a two-way ANOVA, but in all cases this was found to be non-significant so blocking was excluded from the final analyses. Whenever necessary the data were transformed to meet the assumptions of the statistics used. Where this was not possible analogous non-parametric tests were utilized (see figure legends). In all cases where a

parametric analysis was utilised any significant results were followed by Tukeys *post-hoc* analysis.

4.4 Results

4.4.1 Hemiparasite performance

The effects of the heterospecific host treatment on hemiparasite biomass varied considerably depending on the host plant species concerned. When *Rhinanthus minor* was grown with the two leguminous hosts plants, *Lotus corniculatus* and *Ononis repens*, its biomass was marginally, though not significantly, smaller than either homospecific host plant treatment ($F_{2.45} = 2.46$, $P = 0.097$) (Figure 1a). When grown with *L. corniculatus* and the non-leguminous forb *Sanguisorba minor*, *R. minor* plants in the heterospecific host treatment achieved more biomass than both homospecific treatments, although again the difference was not significant (Kruskal-Wallis: $H_2 = 2.93$, $P = 0.231$) (Figure 1b). There was however, a significant difference between the treatments in the experiment with *Dactylis glomerata* (Kruskal-Wallis: $H_2 = 16.11$, $P < 0.001$), where again the hemiparasites grown with heterospecific hosts, *D. glomerata* and *L. corniculatus,* achieved the greatest mean biomass. Hemiparasites attached to two *D. glomerata* plants performed poorly (Figure 1c).

4.4.2 Aphid Performance

Despite the variable performance of *R. minor* on the different host plant combinations, the pattern in the performance of the invertebrate herbivore *Aphis gossypii* feeding on the hemiparasite were remarkably similar, regardless of the second host species grown with *L. corniculatus*. In all three experiments, the number of aphids after 14 days was lowest on *R. minor* in the *L. corniculatus* homospecific

host treatment and highest on the other homospecific host treatments, with the aphids feeding on the heterospecific host treatment performing at an intermediate level. Analysis of the experiment using *L. corniculatus* and *O. repens* showed that, while there was a highly significant difference in aphid performance overall ($F_{2,45}$ = 8.43 P = 0.001), Tukey *post hoc* analysis revealed that the heterospecific host treatment was in fact not significantly different from either homospecific treatment (Figure 2a). In the remaining two experiments (using either *L. corniculatus* and *S. minor*, or *L. corniculatus* and *D. glomerata*), in addition to a highly significant difference between the treatments, *post hoc* analysis revealed that all treatments were significantly different from one-another ($F_{2,52}$ = 57.69, P < 0.001, and $F_{2,50}$ = 47.80, P < 0.001 respectively) (Figure 2a and b).

4.4.3 Nitrogen:Carbon Ratio

The nitrogen carbon (N:C) ratio in the above ground portion of the hemiparasites increased in the presence of a nitrogen fixing leguminous host. In the experiment where both host plants were legumes, there was no significant difference between the treatments ($F_{2,21}$ = 0.87, P = 0.433) (Figure 3a). In the experiment involving the non-leguminous forb, *S. minor* and the grass *D. glomerata* there was a clear gradient, with hemiparasites grown with two legumes having significantly higher N:C ratios than either heterospecific or homospecific treatments (Experiment 1, $F_{2,21}$ = 9.07, P = 0.001; Experiment 3, $F_{2,21}$ = 13.98, P < 0.001), and the homospecific non-leguminous treatments having marginally lower N:C ratio than the heterospecific host treatment (Figures 3a and b).

4.4.4 Haustorial Connections

In the heterospecific host treatment between *L. corniculatus* and *O. repens* it was possible to count a large proportion of the haustorial connections and to assign each of them to one of the hosts. There was a significant negative correlation between the proportion of haustoria attached to *L. corniculatus* and the number of aphids on *R. minor* after 14 days (Pearsons product moment correlation: $r_{2,\ 13}$ = -0.0548, P = 0.034), suggesting that the more connections the hemiparasite formed with *L. corniculatus* the greater the negative impact on its invertebrate herbivores (Figure 4).

4.5 Discussion

Previous work on the hemiparasite *Rhinanthus minor* has shown that the identity of its host can have major impacts on its performance (Seel *et al.*, 1993a, Cameron *et al.*, 2006) and on the performance of its invertebrate herbivores (Chapter 3). It is, however, important to recognise that *R. minor* rarely forms associations with a single host species at any one time (Gibson and Watkinson, 1989). In the current study, I have demonstrated that these additional host associations can have important impacts on the hemiparasite's performance and on its interactions with invertebrate herbivores. This is because the growth and chemical composition of *R. minor* differs depending on whether it attaches to a single host species, or to multiple host species, with knock-on consequences for the herbivores feeding upon it.

In line with the predictions of Govier *et al.* (1967) and the work of Marvier (1998), I found that, in experiments using the legume *Lotus corniculatus* and either the grass *Dactylis glomerata* or the forb *Sanguisorba minor, R. minor* produced slightly more biomass when grown with two different host species than when grown with

homospecific pairs of these hosts. Interestingly however, when *R. minor* was grown with two legume species, *Ononis repens* and *L. corniculatus*, there was a slight, but non-significant, reduction in biomass in comparison with when the hemiparasite was grown with homospecific pairs of either host. These results add weight to the hypothesis of Govier *et al.* (1967) that attaching to different plant species that have subtle differences in their physiology and chemical composition, will provide the parasite with a broader range of nutrients or defensive compounds and thereby enhance its performance. Furthermore, other work on *R. minor* has shown that biomass is closely correlated with the number of flowering structures produced (Chapter 3), suggesting that one advantage to the hemiparasite of attaching to multiple host species is increased reproductive output and hence potentially increased fitness.

In addition to the effects of multiple host plants on the hemiparasite's performance, attachment to more than one host species has important implications for the hemiparasite's invertebrate herbivores. The results from the current study confirm the results from my previous work which showed that performance of aphids feeding on *R. minor* is greatly reduced when the hemiparasites is grown with the host plant *L. corniculatus* (Chapter 3). In addition to this, the results from the current study show that the negative effects on the hemiparasite's invertebrate herbivores conferred by this host plant are still apparent in the presence of a second host species. In two of the three experiments aphid performance was significantly reduced by the presence of one *L. corniculatus* plant compared to their performance on hemiparasites grown without this host plant, with a similar, though non-significant trend in the third. Furthermore, this result was consistent regardless of the identity of the second host plant species.

Work on the hemiparasite *Castilleja wightii* grown with multiple host plants demonstrated that the major factor affecting its herbivores' performance was the percentage of nitrogen in the hemiparasite (Marvier, 1998). Indeed, my previous work on *R. minor* has shown that, for the majority of host-hemiparasite associations, the performance of aphids feeding on *R. minor* is positively correlated with the Nitrogen to Carbon ratio (N:C) of the hemiparasite (Chapter 3). While nitrogen has long been regarded as a major factor controlling invertebrate herbivore performance (Mattson, 1980, White, 1984), and has been shown to be an important factor for the invertebrate herbivore utilized here (Nevo and Coll, 2001), it does not appear to be related to their performance in the current study. Instead, it would appear that when *R. minor* is attached to *L. corniculatus* it is of poor quality as a host for aphids, possibly because *L. corniculatus* passes a defensive compound to the hemiparasite *R. minor*. While the identity of this compound remains unknown there are several potential candidates including a range of nitro-bearing molecules known to be present other species within the *Lotus* genus (Williams, 1983, Gnanasunderam and Sutherland, 1986), and various non-protein amino acids which are common to leguminous plants (Bennett and Wallsgrove, 1994).

Interestingly, it appears that the degree to which *L. corniculatus* indirectly affects the invertebrate herbivores of *R. minor* is related to the proportion of haustorial attachments between the hemiparasite and this host. In most cases, even a relatively low proportion of attachments (~0.2-0.3) to *L. corniculatus* appears to have a negative effect on aphid performance. Although the negative impact of attachment to *L. corniculatus*, is maintained in the presence of a second host, the relative abundance, size and density of these hosts are all likely to have important implications for these indirect effects. While the negative indirect effects on

herbivores seen in the current study focus on the host plant *L. corniculatus*, it would seem likely that numerous other host plant species could confer herbivore resistance to the hemiparasite *R. minor*, and these are also likely to be moderated, perhaps in unpredictable ways, by the presence of other host plant species.

It has been known for some time that the hemiparasite *R. minor* can form haustorial connections with a wide variety of host plants (Gibson and Watkinson, 1989), and can moderate competition within plant communities (Gibson and Watkinson, 1991). Furthermore, there is extensive evidence that these interactions have major impacts on plant community composition (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Bullock and Pywell, 2005, Gibson and Watkinson, 1992). Because of this and the highly dynamic nature of *R. minor* population cycles (Cameron *et al.*, 2009), the hemiparasite is likely to be faced with a largely unpredictable set of potential host plants. Several factors are known to affect the performance of hemiparasitic plants (host age (Seel and Press, 1996), host damage (Salonen and Puustinen, 1996, Puustinen and Salonen, 1999), host-hemiparasite proximity (Keith *et al.*, 2004), and growing media (Salonen and Puustinen, 1996, Cameron *et al.*, 2009)). Whilst these are likely to add further complexity to the interactions of this hemiparasite, this study adds considerably to our understanding of how *R. minor* copes, and indeed thrives, under such variable conditions.

4.6 Figures

Figure 1a.

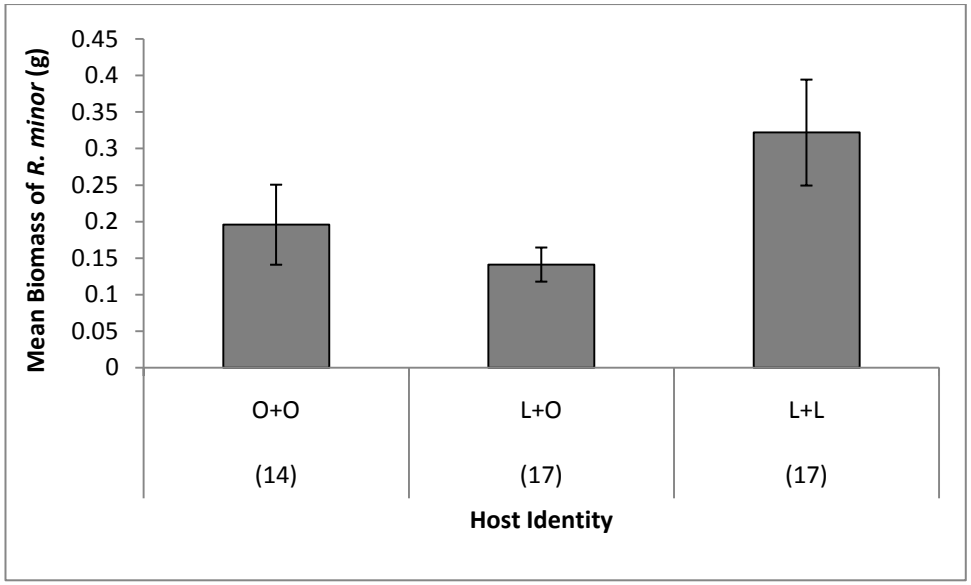

Figure 1b.

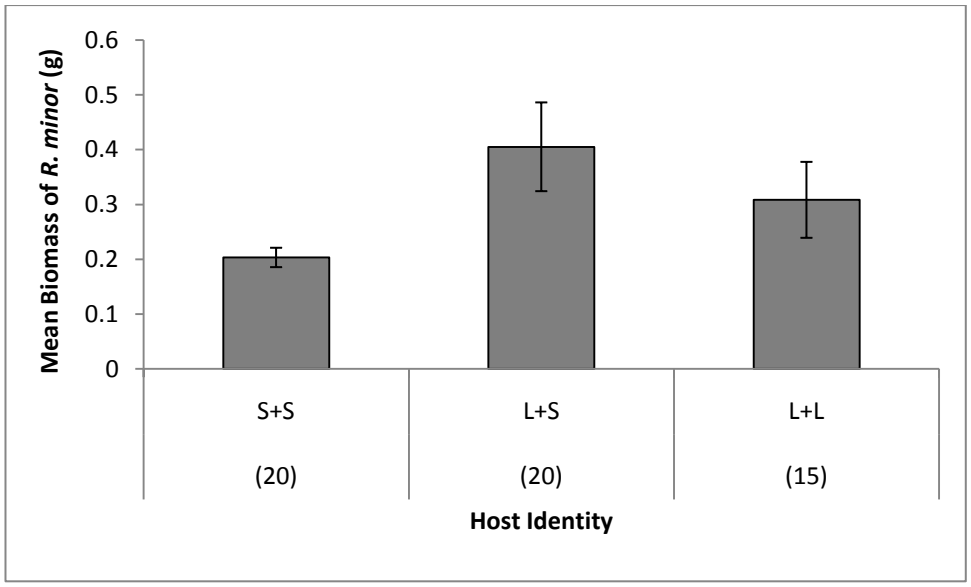

Figure 1c.

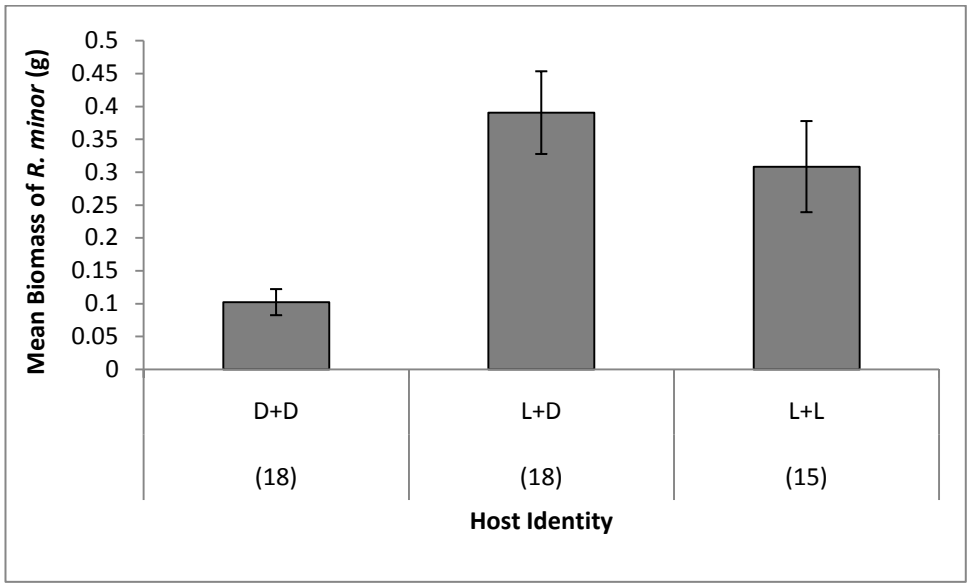

Figure 1a, b and c. Mean biomass (g) achieved by *Rhinanthus minor* when grown with pairs of host plants. **1a**. Host plant treatments were paired combinations of *Ononis repens* (O+O) or *Lotus corniculatus* (L+L), or one of each host plant per pot (L+O). One way ANOVA: $F_{2.45}$=2.46, P=0.097. Values were square root transformed to meet the assumptions of the test statistic. **1b**. Host plant treatments were paired combinations of *Sanguisorba minor* (S+S) or *L. corniculatus* (L+L), or one of each host plant per pot (L+S). Kruskal-Wallis: H_2 = 2.93, P = 0.231. **1c.** Host plant treatments were paired combinations of *Dactylis glomerata* (D+D) or *L. corniculatus* (L+L), or one of each host plant per pot (L+D). Kruskal-Wallis: H_2 = 16.11, P < 0.001. Error bars show ± 1 SEM, and the replicate number is shown in parentheses below each treatment.

Figure 2a.

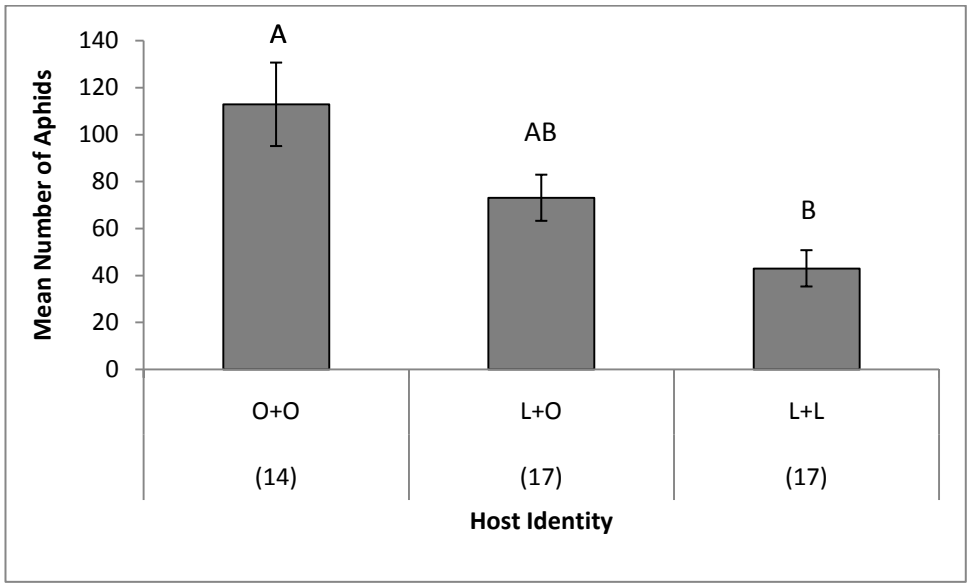

Figure 2b.

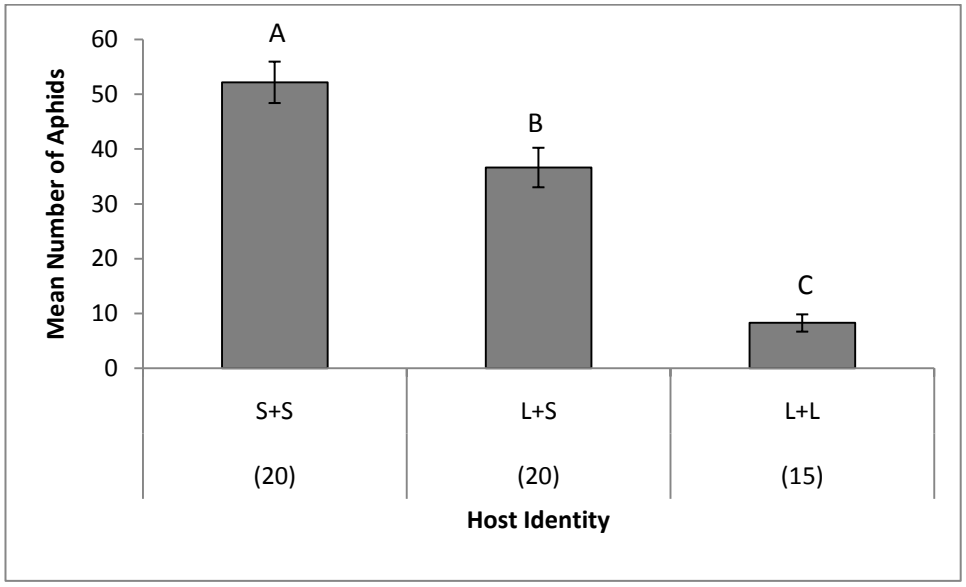

Figure 2c.

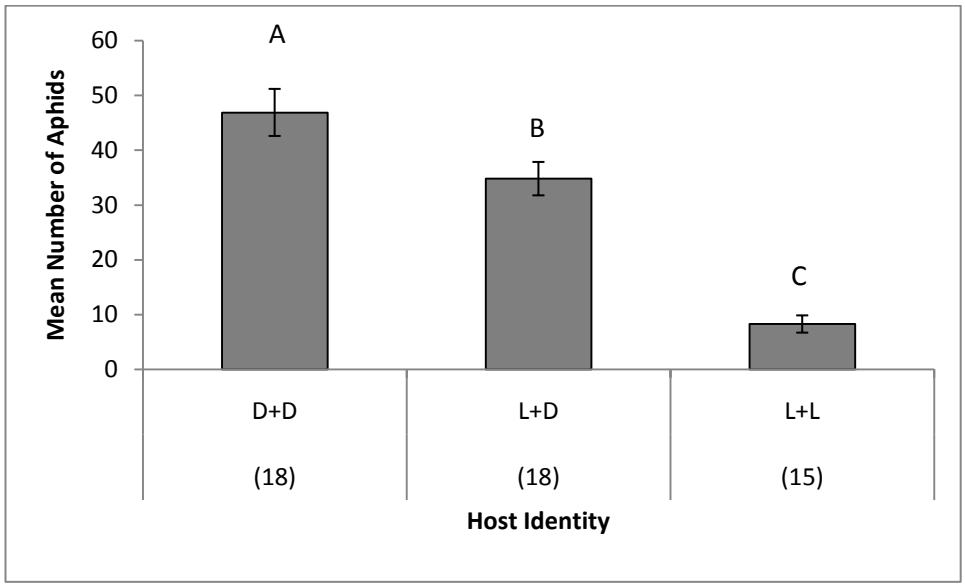

Figure 2a, b and c. The mean number of aphids after 14 days on *R. minor* plants grown with two host plants (starting density = 20 aphids). **1a**. Host plant treatments were paired combinations of *Ononis repens* (O+O) or *Lotus corniculatus* (L+L), or one of each host plant per pot (L+O). One-way ANOVA: $F_{2,45} = 8.43$ $P = 0.001$. **1b**. Host plant treatments were paired combinations of *Sanguisorba minor* (S+S) or *L. corniculatus* (L+L), or one of each host plant per pot (L+S). One-way ANOVA $F_{2,52}= 57.69$, $P=<0.001$. Data were transformed using the Freeman Tukey transformation equation $(((\sqrt{\chi}) + (\sqrt{\chi + 1})) / 2)$ to meet the assumptions of the test statistic. **1c.** Host plant treatments were paired combinations of *Dactylis glomerata* (D+D) or *L. corniculatus* (L+L), or one of each host plant per pot (L+D). One way ANOVA $F_{2,50} = 47.80$, $P=<0.001$. Data were transformed using the Freeman Tukey transformation equation (see above). Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis $P=0.05$). Error bars show ± 1 SEM, and the replicate number is shown in parenthesis below each treatment.

Figure 3a

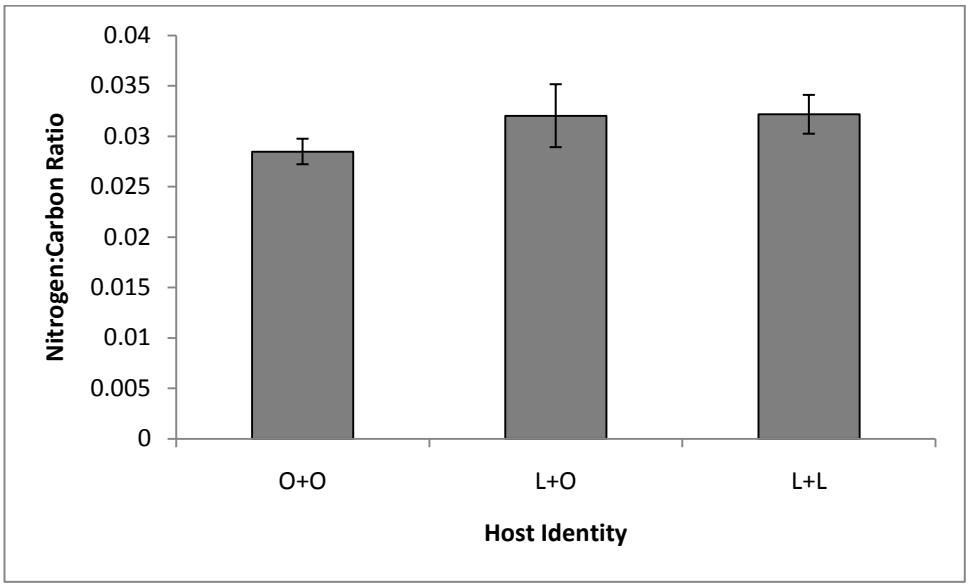

Figure 3b.

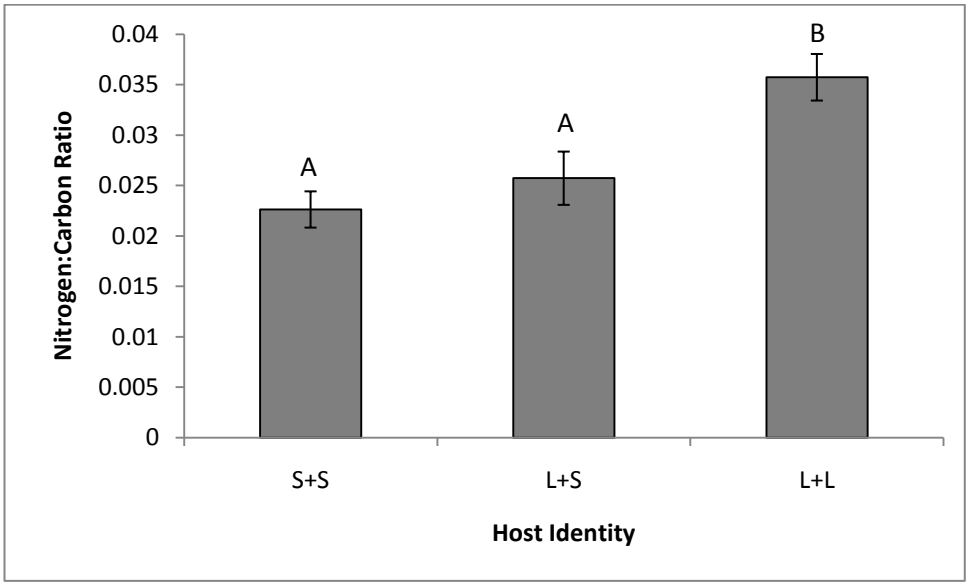

Figure 3c.

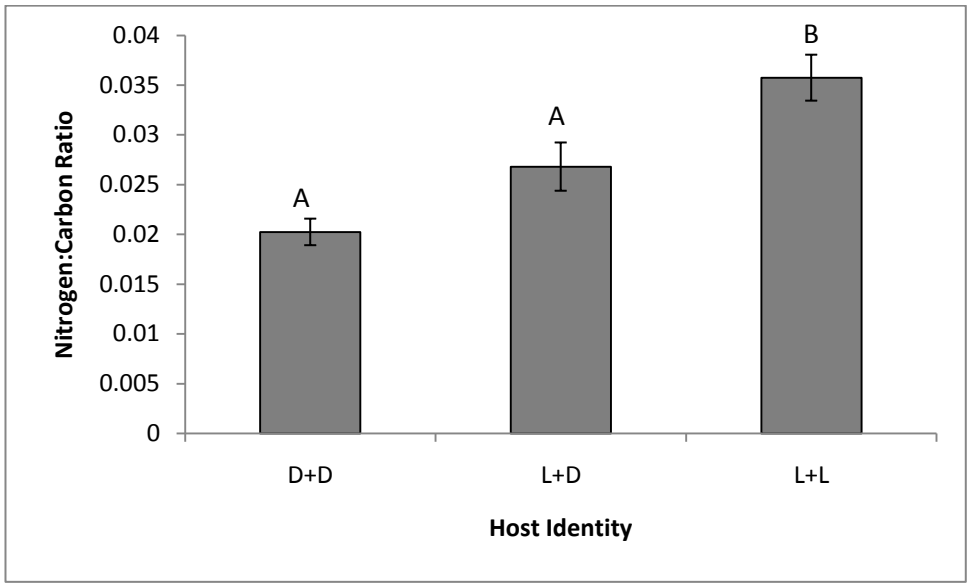

Figure 3a, b and c. Mean Nitrogen to Carbon ratio of *Rhinanthus minor* plants when grown with various host species. **1a**. Host plant treatments were paired combinations of *Ononis repens* (O+O) or *Lotus corniculatus* (L+L), or one of each host plant per pot (L+O). One-way ANOVA, $F_{2,21} = 0.87$, $P = 0.433$. **1b**. Host plant treatments were paired combinations of *Sanguisorba minor* (S+S) or *L. corniculatus* (L+L), or one of each host plant per pot (L+S). One-way ANOVA, $F_{2,21} = 13.98$, $P < 0.001$. **1c.** Host plant treatments were paired combinations of *Dactylis glomerata* (D+D) or *L. corniculatus* (L+L), or one of each host plant per pot (L+D). One-way ANOVA, $F_{2,21} = 13.98$, $P < 0.001$. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis $P=0.05$). Error bars show ± 1 SEM (in all cases n=8).

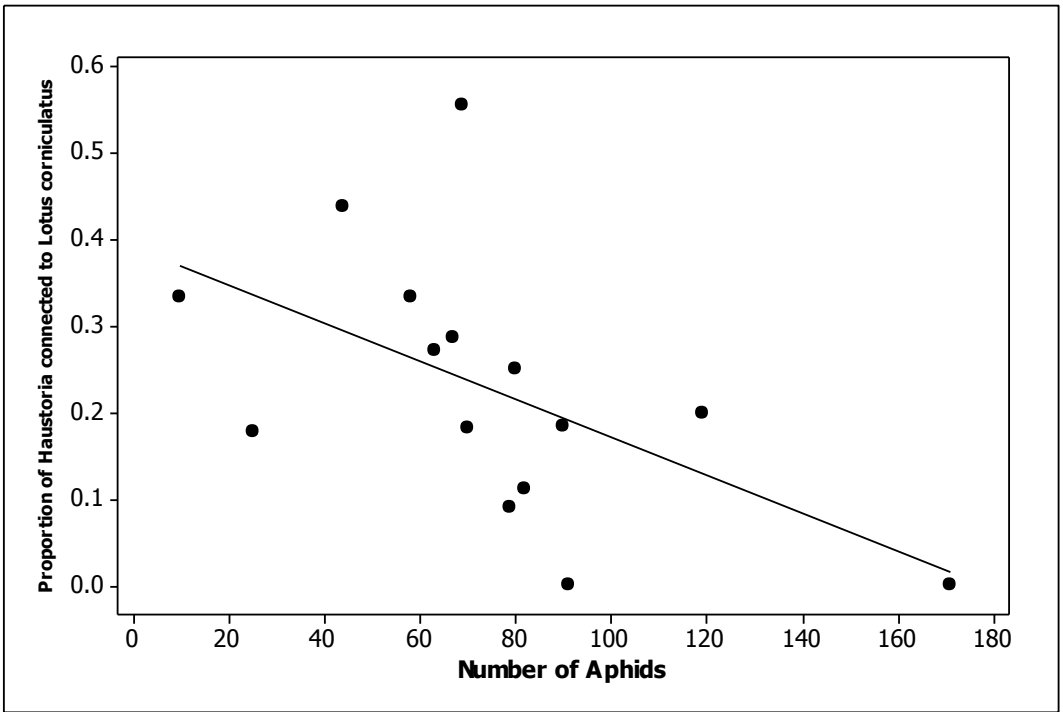

Figure 4. Scatterplot showing the proportion of haustorial attachments between *Rhinanthus minor* and *Lotus corniculatus* against the number of aphids on R. *minor* after 14 days (starting density of 20) when the parasite was grown with both *L. corniculatus* and *Ononis repens*. Pearson product moment correlation: $r_{2,\ 13}$ = -0.0548, P = 0.034. Regression line, y = -0.2204 x + 39.19.

Chapter 5. Host Damage and Nutrient Addition Indirectly Affect the Performance of Invertebrate Herbivores Feeding on the Hemiparasite *Rhinanthus minor*

5.1 Abstract

The hemiparasite *Rhinanthus minor* is a common component of many northern temperate grasslands. It can have major impacts on ecosystem processes, and is often present at very high densities, therefore constituting an important potential food source for invertebrate herbivores. *R. minor* is known to form parasitic attachments with a wide variety of host plants and there is considerable variation in the effects of these hosts on the hemiparasite, and on the performance of its invertebrate herbivores. Factors affecting the growth and chemical composition of the host plant, such as soil nutrient levels or herbivory might also have dramatic effect on the performance of the hemiparasite, and could further influence the impact of the host on *R. minor*. These factors are also likely to have major indirect consequences for the invertebrate herbivores of the hemiparasites by altering *R. minor's* nutrient content and its levels of defensive compounds. The aim of the this study was to investigate the effects of damage and nutrient addition to the host plant on the invertebrate herbivores of the hemiparasite *R. minor* attached to this host using two contrasting host plant species.

The two host species were both 'good' hosts for the hemiparasite, but had highly contrasting indirect effects on the performance of the hemiparasite's invertebrate herbivores. While neither factor had any significant effect on the performance of the hemiparasite, both the addition of nutrient and host plant damage differentially

affected the hemiparasite's herbivores. Interestingly, both factors reduced the performance of aphids feeding on *R. minor* attached to the host which was previously beneficial to aphid performance, and increased the performance of aphids associated with the host which was previously most detrimental to their performance. Thus, the strong indirect effects of host identity on aphid performance were largely removed by the impacts of both nutrient addition and host damage.

Key words: Hemiparasite; *Rhinanthus minor*; host parasite interaction; invertebrate herbivores; C:N ratio; indirect interaction; fertilizer; mechanical damage.

5.2 Introduction

Parasitic plants can have major effects on ecosystem processes, manipulating nutrient cycles (Quested *et al.*, 2003b, Quested *et al.*, 2003a, Quested, 2008, Bardgett *et al.*, 2006), microbial communities (Bardgett *et al.*, 2006), invertebrate herbivores (Puustinen and Mutikainen, 2001) and plant community structure (Joshi *et al.*, 2000, Pywell *et al.*, 2004, Bullock and Pywell, 2005, Bullock *et al.*, 2007, Davies *et al.*, 1997, Gibson and Watkinson, 1992, Ameloot *et al.*, 2005, Ameloot *et al.*, 2008, Pennings and Callaway, 1996). Parasitic plants are thought to be present in most terrestrial plant communities (Musselman and Press, 1995) and make up around 1% (in excess of 3000 species) of all known flowering plants species (Nickrent *et al.*, 1998, Atsatt, 1983). They have the potential to have a profound impact on the communities in which they are found.

The common grassland hemiparasite *Rhinanthus minor*, like all other root hemiparasites, attaches to the root system of its host by means of specialised organs known as haustoria (Kuijt, 1969, Riopel and Timko, 1995). These parasitic attachments allow the hemiparasites to remove solutes, mainly in the form of amino acids and other nutrients, directly from the host's xylem stream (Jiang *et al.*, 2004b). *R. minor* can form haustorial connections with around 50 species from 18 different plant families (Gibson and Watkinson, 1989). Numerous studies have demonstrated that not all of these potential host plants are equally good for the parasite's performance (Seel *et al.*, 1993a, Cameron *et al.*, 2006, Seel and Press, 1993). For example, some host plants can actively defend their xylem stream from the hemiparasite via lignification or localized die back and thereby effectively block the flow of solutes (Cameron *et al.*, 2006, Rumer *et al.*, 2007, Cameron and Seel, 2007). While many other host plant species do not appear defend themselves physically, there is still considerable variation in the performance of the hemiparasite attached to these hosts (Seel *et al.*, 1993a). It would seem likely that this variation is due in part to the amount of nitrogen the hemiparasite is able to access from different host plants (Seel *et al.*, 1993a, Press, 1995, Seel and Press, 1993) and because of differences in the types and quantities of compounds passed from the host plant to the hemiparasite (Govier *et al.*, 1967).

In addition to variation caused by the identity of the host, several other factors have been shown to affect hemiparasite performance indirectly. For example, Davies and Graves (1998) found that *R. minor* plants attached to the host *Lolium perenne* produced more biomass and more flowers if the grass was simultaneously infected by arbuscular mycorrhizal fungi. Puustinen and Salonen (1999) and Salonen and Puustinen (1996) showed that host plant defoliation had a significant effect on the

hemiparasite biomass, although these effects were both time and host plant specific. In addition, soil type (Salonen and Puustinen, 1996), fertilizer addition (Davies and Graves, 2000, Seel *et al.*, 1993b) and host plant proximity (Keith *et al.*, 2004) have all been shown to have major effects on hemiparasite performance. All previous findings suggest that the nutritional status of the host plant is likely to influence hemiparasite performance strongly.

R. minor represents an important and substantial food source for invertebrate herbivores, making up as much as 8% of the available above ground biomass at certain times of the year (Davies *et al.*, 1997). Unlike many plants, the quality of a hemiparasite as a source of food for insect herbivore is critically dependent on the identity of the hemiparasite's host (Adler *et al.*, 2001, Adler, 2000, Adler, 2002, Adler, 2003, Marvier, 1996, Marvier, 1998). This governs both the nutrient content of the parasite (Seel *et al.*, 1993a) and its levels of defensive compounds, which are often obtained from the host (Adler and Wink, 2001, Wink and Witte, 1993, Rasmussen *et al.*, 2006, Stermitz *et al.*, 1989, Lehtonen *et al.*, 2005) . These transfers of nutrient and secondary compounds from the host plant can make the parasites an unpredictable resource for herbivores, and may help to explain why relatively few herbivores choose to specialise on them (Lehtonen *et al.*, 2005).

The rate of nutrient acquisition by the hemiparasite from its host will largely be driven by the nutrient status of the host (Seel *et al.*, 1993a). Therefore, factors which increase this are likely to increase the quality of the hemiparasite for the insects feeding on it. The rate of acquisition of defensive compounds from the host is harder to predict, but damage to the host plant is likely to increase its levels of secondary compounds (Karban and Myers, 1989). This may result in increased transfer of these

compounds to the hemiparasite and consequently a decrease in the performance of the herbivores feeding on it.

The aim of this study was to investigate how factors which increase or decrease the nutrient quality of the host plant impact on the performance of herbivores feeding on the hemiparasite attached to this host. Specifically, I tested how host plant damage and increases in nutrient levels indirectly affect the performance of *R. minor's* invertebrate herbivores. I examined the performance of the generalist aphid *Aphis gossypii,* Glover (Homoptera: Aphididae), feeding on the hemiparasite *R. minor.* Hemiparasites were grown with one of two host plant species, *Lotus corniculatus* or *Ononis repens.* Previous work on the interaction between these plants species has shown that these two plants are both 'good' hosts for *Rhinanthus minor* but have very different indirect effects on the performance of the hemiparasite's invertebrate herbivores (Chapter 3). *L. corniculatus* greatly reduces the performance of aphids feeding on *R. minor* attached to it, potentially because it passes a defensive compound to the hemiparasite, while aphids perform particularly well when feeding on hemiparasites attached to *O. repens* (Chapter 3). Thus, both species represent good hosts for *R. minor* but have very different consequences for the aphids feeding on the hemiparasite. Therefore, manipulating nutrient availability (via fertiliser addition) and the levels of defensive secondary compounds (via mechanical damage) in these host plants will allow me to test the potential mechanisms underpinning the variation in the performance of the aphids on *R. minor* attached to different host plants.

The hypotheses being tested were therefore:

1. Increasing the nutrient availability will increase the growth and performance of both the host and the hemiparasite.
2. Increasing the nutrient availability will increase the performance of invertebrate herbivores feeding on hemiparasites attached to *Ononis repens* due to the increased nutrient content of the hemiparasite.
3. Increasing the nutrient availability will decrease the performance of invertebrate herbivores feeding on hemiparasites attached to *Lotus corniculatus* due to increases in the levels of secondary metabolites the hemiparasite removes from its host.
4. Host plant damage will lead to a decrease in the performance of aphids feeding on hemiparasites attached to the host plant *Ononis repens* due to a decrease in nutrient availability.
5. Host plant damage will lead to an increase in the performance of aphids feeding on hemiparasites attached to the host plant *Lotus corniculatus* due to a reduction in the levels of secondary metabolites removed by the hemiparasite.

5.3 Material and Methods

5.3.1 Plant material

Rhinanthus minor seed was collected from Castle Hill National Nature Reserve in East Sussex (Grid ref: TQ 375 065) in late July and dried at ambient room temperature. Host plant seeds (purchased from Emorsgate Seeds, Norfolk) were from the same source and batch as those used in my previous experiment (Chapter 3). All seeds were stored in sealed containers with silica gel at 4°C until required.

Prior to stratification *R. minor* seeds were surface sterilised using 5% household bleach for 60 seconds and then washed four times in sterile water. Approximately 90 seeds were then placed onto 9cm petri dishes containing a damp layer of sterile Whatman glass fibre filter paper and capillary matting. Petri dishes were then sealed with parafilm and placed in a refrigerated room at 4°C for 84 days.

All plant material was grown in greenhouses at the University of Sussex. The temperature was maintained at 15-25°C with supplementary lighting (400W, high pressure sodium lamps) on a 16:8 light:dark regime. Plants were supplied with tap water *ad libitum*. Host plants were germinated on damp vermiculite 28 days after *R. minor* seed stratification had begun. After a further 14 days host plant seedlings were transplanted into 9 cm pots containing six parts sand and one part John Innes No. 2 potting compost. 42 days later, five germinating *R. minor* seedlings were transplanted into each pot. Hemiparasite seedlings were thinned to one per pot when the majority showed signs of attachment (Klaren and Janssen, 1978); this was approximately 21 days after *R. minor* seedlings were transplanted.

5.3.2 Experimental Design

Two treatment regimes were imposed to investigate independently the effect of nutrient addition and the effect of host damage. In both experiments two host plants, *Ononis repens* and *Lotus corniculatus*, were used. The two experiments were conducted simultaneously and arranged in randomised blocks each containing one of each of the experimental treatments (see below).

The first of the two treatments regimes was designed to investigate the effects of increased nutrient availability on the performance of the aphid, *Aphis gossypii*,

feeding on the hemiparasite. This was achieved by the addition of 50ml of either 0.5 or 0.25 regular strength Hoagland's solution twice a week. Two concentrations of the fertilizer were used to investigate if increasing levels of key nutrients had an increasingly strong effect on the insect herbivores. To avoid any potential effects of fertilizer on attachment success and the possibility of host plants outcompeting the hemiparasites seedlings for light (Keith *et al.*, 2004), fertiliser application was commenced when the hemiparasites showed signs of attachment (Klaren and Janssen, 1978) (approximately 21 days after hemiparasites were transplanted). The treatments therefore consisted of (i) pots receiving two weekly additions of 50ml of 0.5 regular strength Hoaglands solution, (ii) pots receiving 50ml of 0.25 regular concentration Hoaglands twice weekly, and (iii) pots that received 50ml of water twice weekly. These three separate treatments were applied to both host plant/hemiparasite combinations giving a total of six treatments for this experiment.

The second treatment regime was designed to investigate the effect of host plant damage on the performance of the aphid, *Aphis gossypii*, feeding on the hemiparasite. Host plant damage was achieved by mechanically cutting approximately 25 percent of the host plant's leaves in half at 90 degrees to their midrib once a week. As in the first experiment, host plant damage was commenced when the majority of hemiparasites showed signs of attachment. The treatments therefore consisted of (i) pots containing hosts and hemiparasites and in which the host plant received weekly damage to approximately 25 percent of their leaves, and (ii) pots containing host and hemiparasites that received no damage. These treatments were applied to both host plant species/hemiparasite combinations giving a total of four treatments for this experiment.

5.3.3 Hemiparasite and Aphid Performance

Cultures of *Aphis gossypii* were reared on *R. minor* plants grown in trays containing two potential host plants, *Trifolium pratense* and *Holcus lanatus* so therefore had no prior indirect exposure to the hemiparasite's experimental host plants. 70 days after the germinating *R. minor* seeds were introduced to the host plants, 20 aphids were caged onto the surviving hemiparasites using specifically designed clear plastic vented tubes, which enclosed the hemiparasite. The aphids were then left to multiply for 14 days, at which point the aphids were counted and the above ground portions of both the host and the hemiparasite were harvested, dried at 60°C for 2 days and weighed.

5.3.4 Nitrogen : Carbon Ratio

The nitrogen to carbon (N:C) ratios of the above ground portion of the hemiparasites was determined in order to assess if the nitrogen concentration of the hemiparasite plant material was affected by host plant damage and nutrient addition, and whether this in turn affected aphid population growth. This was achieved via flash combustion and chromatographic separation of approximately 1.5mg of ground and homogenised plant material, calibrated against a standard compound ($C_{26}H_{26}N_2O_2S$), using an elemental combustion system (Costech Instruments, Milan, Italy).

5.3.5 Statistical Analysis

All statistical analyses were done using Minitab 15 for Windows (Minitab, Inc). In Experiment 1, aphid performance, *R. minor* biomass and N:C ratios were analysed using a two-way ANOVA and Tukey *post-hoc* analysis, with nutrient addition and host plant as the two independent variables. Treatment effects on the biomass of each of the two host plants were assessed using one-way ANOVA. In the second

experiment, in which the host plants were mechanically damaged, the performance of the aphids, *R. minor* biomass, N:C ratios of the hemiparasites and the biomass of the host plants were analysed using two-sample t-tests. When required, the data were either square root or log10 transformed to meet the assumptions of the statistical test.

5.4 Results

5.4.1 Nutrient Addition

Nutrient addition had a highly significant effect on the biomass of the host *Ononis repens* ($F_{2,55}$ = 31.98, P < 0.001). Unsurprisingly, increased nutrient addition lead to increased host biomass, with plants receiving 0.5 concentration Hoagland's solution achieving significantly more biomass than either control plants or those receiving 0.25 concentration Hoagland's solution (mean biomass of the host receiving: (i) 0.5 Hoaglands, 10.20 ± 0.39g; (ii) 0.25 Hoaglands, 7.03 ± 0.45g and (iii) control, 5.61 ± 0.36.) (Tukeys *post-hoc* analysis P = 0.05). The addition of nutrients had a marginally significant effect on the biomass of *Lotus corniculatus* ($F_{2,60}$ = 3.00, P = 0.057), and again showed a trend towards increasing biomass with increasing nutrient addition (mean biomass of the host receiving: (i) 0.5 Hoaglands, 16.35 ± 0.89g; (ii) 0.25 Hoaglands, 14.46 ± 0.88g and (iii) control, 13.57 ± 0.63).

Neither nutrient addition nor host identity had a significant effect on the biomass of the hemiparasite (two-way ANOVA; nutrient addition, $F_{2,115}$ = 1.80, P = 0.170; host identity $F_{1,115}$ = 0.15, P = 0.696; interaction $F_{2,115}$ = 0.80, P = 0.450), although there was a slight increase in biomass with increasing nutrient addition (Figure 1). There was no effect of nutrient addition on Nitrogen to Carbon (N:C) ratio of the hemiparasites ($F_{2,116}$ = 1.20, P = 0.305). Host plant identity, however, did have a strong effect on the N:C ratio of the hemiparasites ($F_{1,115}$ = 21.52, P < 0.001), but

there was no significant interaction between the two factors ($F_{2,116}$ = 1.29, P < 0.275). *Post-hoc* analysis revealed that hemiparasites attached to *O. repens* in all treatments had significantly lower N:C ratios than hemiparasites attached to *L. corniculatus* receiving 0.5 strength fertilizer, but there were no other significant differences (Tukey *post-hoc* analysis, P = 0.05) (Figure 2).

Host identity had a strong effect on the performance of aphids (in terms of change in number) feeding on *R. minor* (two-way ANOVA; host identity, $F_{1,115}$ = 31.28 P < 0.001), but overall, there was no significant effect of nutrient addition ($F_{2,115}$ = 0.15 P = 0.861). There was a strong interaction between the two factors ($F_{2,115}$ = 6.28 P = 0.002). Aphid numbers increased most on hemiparasites attached to *O. repens* control plants, but their performance was significantly lower on hemiparasites grown with *O. repens* receiving 0.25 strength fertiliser (Tukey *post-hoc* analysis P = 0.05). Conversely, aphid performance was lowest on *R. minor* plants attached to *L. corniculatus*, but slightly increased with the addition of nutrients. The result of these contrasting indirect effects is that, whilst there was a significant host plant effect on aphid performance when no nutrients were added, this difference disappeared when additional nutrients were applied (Figure 3).

5.4.2 Host Plant Damage

The biomass of *L. corniculatus* was significantly reduced by mechanical clipping (t_{38} = -3.29, P = 0.002) (mean biomass of the (i) damaged = 10.22g ± 0.81g and (ii) undamaged = 13.57 ± 0.63g). Furthermore, while host plant clipping lead to a marginal decrease in hemiparasite biomass (Figure 4) and an increase in hemiparasite N:C ratio (Figure 5), neither factor was significant (biomass: t_{38} = -0.73, P= 0.470; N:C ratio: t_{38} = -1.50, P = 0.142). Host clipping did, however,

significantly increase the performance of aphids feeding on the hemiparasite (t_{39} = -2.48, P = 0.018) (Figure 6).

Mechanical clipping did not significantly reduce the biomass of the host *O. repens* (mean biomass of (i) damaged = 5.46 ± 0.39g, and (ii) undamaged = 5.61 ± 0.36g. t_{36} = -0.29, P = 0.773), presumably because of compensatory growth. Furthermore, host plant damage did not have any significant indirect effect on hemiparasite biomass (t_{36} = 0.61, P = 0.544) (Figure 4) or hemiparasite N:C ratios (t_{36} = -0.63, P = 0.534) (Figure 5), nor did it significantly affect the performance of the aphids (t_{36} = 1.90, P = 0.065). There was, however, a slight reduction in the performance of aphids feeding on parasites attached to damaged host plants compared to the controls (Figure 6).

5.5 Discussion

It has been long been established that host identity can have major impacts on the performance of the hemiparasite *Rhinanthus minor* (Seel *et al.*, 1993a, Seel and Press, 1993, Cameron *et al.*, 2006). The indirect effects of these impacts on the invertebrate herbivores feeding on *R. minor* remain poorly understood. This study demonstrates that these indirect indirections can be significantly modified by simulated herbivory of the hemiparasite's host plant and by the addition of nutrients.

Perhaps unsurprisingly, I found that the addition of nutrients increased the biomass of both host plants, producing a highly significant positive effect on *Ononis repens*. Hemiparasite biomass showed only very marginal increases with the addition of nutrients. These marginal increases are extremely modest in comparison to the

results observed elsewhere (Seel *et al.*, 1993b, Davies and Graves, 2000, Salonen and Puustinen, 1996), but there are several potential explanations for this. Firstly, the relatively late application of nutrients may have reduced the impacts on the hemiparasite. Secondly, the nutrient level in the growing media may not have been a limiting factor for the host plants and hemiparasites. The responses shown by the host plants make these explanations seem unlikely. An alternative explanation lies with the choice of host plants used in these studies. Previous work on this hemiparasite has shown that at very high nitrogen levels *R. minor* displays a non-linear relationship between nitrogen accumulation and photosynthetic rates (Seel *et al.*, 1993a). In previous studies where nutrient levels or growing media were manipulated, the host plants were universally grasses, while in the current study both host plants were nitrogen rich legumes which are known to be highly beneficial to the performance of *R. minor* (Seel *et al.*, 1993b, Seel and Press, 1993). It seems possible therefore, that the hemiparasites were already growing at a near optimal rate, and therefore, the addition of nutrient had only a very marginal impact on their biomass.

Host identity had a significant effect on both the performance of the aphids feeding on the hemiparasites, and on the nitrogen to carbon (N:C) ratio of the hemiparasite, but the addition of nutrients did not significantly affect either of these parameters. The interaction between host identity and fertiliser addition resulted in the performance of the aphids feeding on the hemiparasite attached to the two hosts, *Lotus corniculatus* and *O. repens*, being significantly differentially affected. While the addition of nutrients led to a clear, although statistically non-significant, increase in the performance of aphids feeding on *R. minor* attached to *L. corniculatus*, it caused a significant reduction in the performance of aphids feeding on hemiparasites

grown with *O. repens*. Interestingly therefore, while the aphids feeding on hemiparasites attached to these host plants show highly divergent responses when no additional nutrients are provided, the addition of a fertiliser appears to remove this host identity effect, resulting in there being no significant difference between the treatments in terms of aphids performance.

Because of the limited ability of *R. minor* to access nutrients from their growing media (with the notable exception of phosphorus) (Seel *et al.*, 1993b) it seems likely that these changes in aphid performance are due to the impacts of additional nutrients on the host plant. It is well established that increases in nutrient availability can affect photosynthetic rates, growth allocation (Evans, 1989, Chapin, 1980, Bazzaz *et al.*, 1987) and, importantly, the levels of anti-herbivore defences (Bazzaz *et al.*, 1987). Predicting the responses of host plants to increased nutrient supply is, however, complicated because of the impact of the hemiparasite on the host. *R. minor* is known to remove as much as 20% of the host's water and xylem-borne nutrients (Jiang *et al.*, 2004a, Jiang *et al.*, 2004b, Jiang *et al.*, 2003), and can have a dramatic effect on the host plant's physiology and biochemistry (Stewart and Press, 1990, Graves, 1995). Furthermore, because *R. minor* only has access to the host's xylem (Kuijt, 1969, Riopel and Timko, 1995), only factors affecting this vascular stream would be passed to the hemiparasites.

Mechanical damage of the host plants resulted in a significant reduction in the biomass of *L. corniculatus,* but almost no reduction in the biomass of the *O. repens*. This disparity could be due to differences in biomass allocations between the leaves and stems of the two species, or because *O. repens* compensated for the lost biomass. Host damage had no significant effect on the biomass of the hemiparasite

irrespective of the host's identity. Other studies have shown that host plant damage can cause significant reduction in the biomass of hemiparasites, but these effects are time and species-specific, and vary according to the intensity of the damage (Puustinen and Salonen, 1999, Salonen and Puustinen, 1996). Despite the lack of impact on *R. minor* biomass, host plant damage did have a strong impact on the performance of aphids feeding on the hemiparasite. In particular, the performance of aphids feeding on hemiparasites attached to *L. corniculatus* was increased significantly by host damage, whereas the performance of aphids feeding on the hemiparasites attached to *O. repens* was reduced by the host plants damage. These effects may reflect the small (though non-significant) changes in the hemiparasite N:C ratios, which are known to affect aphid performance (Nevo and Coll 2001; White 1984). In turn, the changes in N:C ratios may result from the host attempting to produce compensatory growth (McNaughton, 1983). It is also possible that the impacts of damage may have had specific effects on types and concentrations of defensive secondary metabolites within the host (Karban and Myers, 1989, Bennett and Wallsgrove, 1994). Such changes could account for the marginally significant decreased performance of aphids feeding on hemiparasites attached to damaged *O. repens* plants.

Plant damage from herbivores and the input of nutrients from urine, dung and other decaying matter are all common and important components of grassland ecosystem processes. This study has shown that factors such as these can indirectly affect the interactions between host plants and the hemiparasite *R. minor,* having major and important knock-on effects for the performance of insect herbivores of *R. minor*. Interestingly, the performance of aphids feeding on hemiparasites attached to the two unmanipulated hosts was highly divergent, with the host plant *O. repens* being

particularly good for aphid performance, whereas *L. corniculatus* was particularly bad. The effects of both nutrient addition and damage increased the performance of aphids feeding on hemiparasites attached to *L. corniculatus*, while decreasing the performance of aphids feeding on hemiparasites attached to *O. repens*. The overall effect of these treatments therefore is to remove the highly divergent impacts of host identity on the performance of the hemiparasite's invertebrate herbivores. While these differential impacts may reflect slight changes in the N:C ratios of the hemiparasites, the exact mechanisms underlying these interactions certainly require further investigation.

5.6 Figures

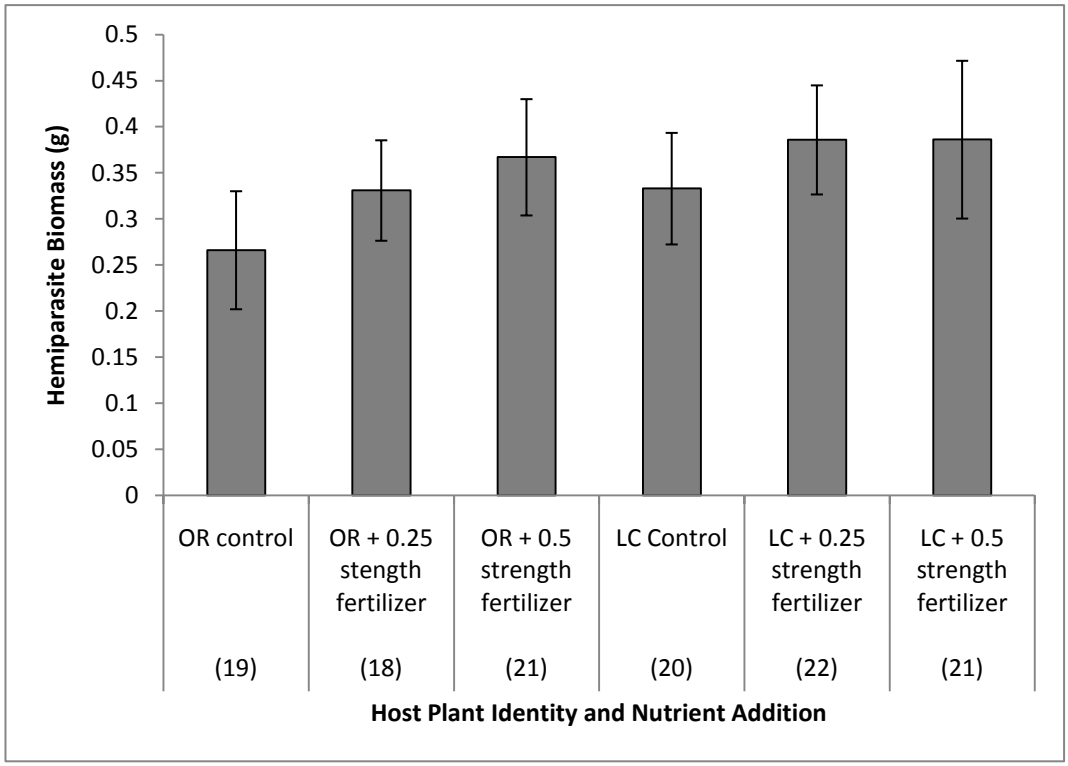

Figure 1. The mean biomass (g) of *Rhinanthus minor* grown with either *Lotus corniculatus* (LC) or *Ononis repens* (OR), and receiving either no additional fertilizer (control), 0.25 strength Hoagland's solution or 0.5 strength Hoagland's solution. Error bars show ± 1 SEM. Replicate number is shown in parenthesis below each treatment. Two-way ANOVA; nutrient addition, $F_{2,115} = 1.80$, $P = 0.170$; host identity $F_{1,115} = 0.15$, $P = 0.696$; interaction $F_{2,115} = 0.80$, $P = 0.450$. Data were $\log_{10}$ transformed to meet the assumptions of the test.

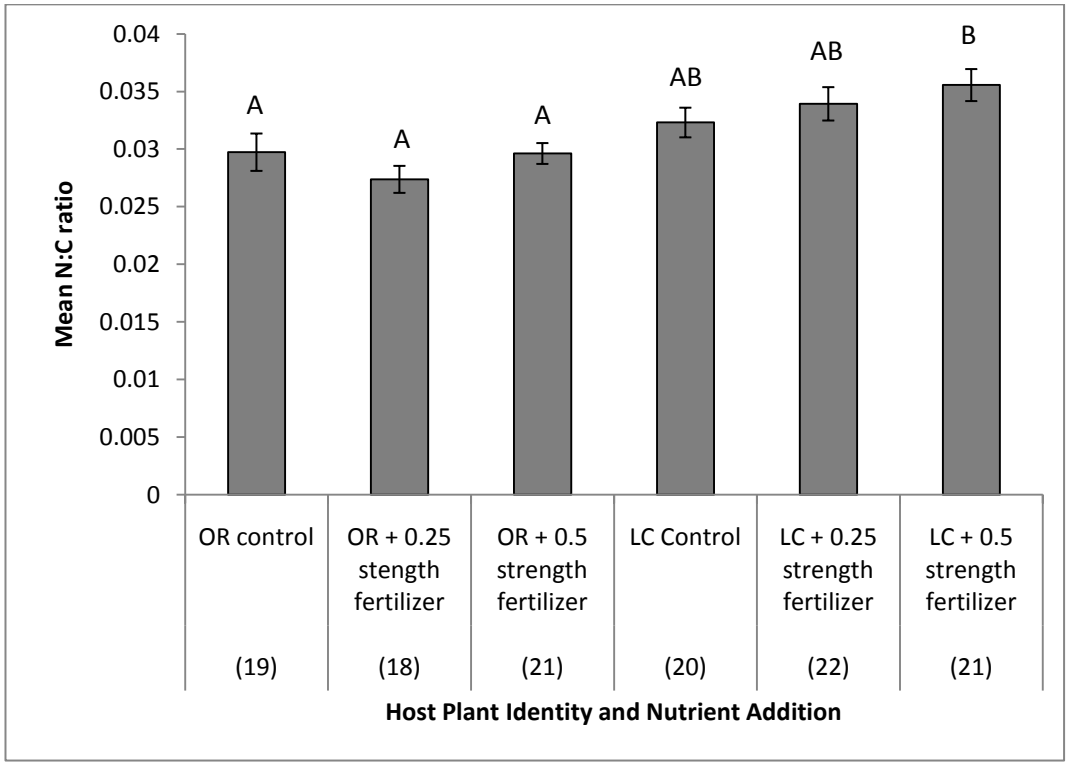

Figure 2. The mean Nitrogen to Carbon ratios of *Rhinanthus minor* grown with either *Lotus corniculatus* (LC) or *Ononis repens* (OR), and receiving either no additional fertilizer (control), 0.25 strength Hoagland's solution or 0.5 strength Hoagland's solution. Error bars show ± 1 SEM. Replicate number is shown in parenthesis below each treatment. Two-way ANOVA; nutrient addition, $F_{2,116}$ = 1.20, P = 0.305; host identity, $F_{1,115}$ = 21.52, P < 0.001; interaction, $F_{2,116}$ = 1.29, P = 0.275. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis P = 0.05)

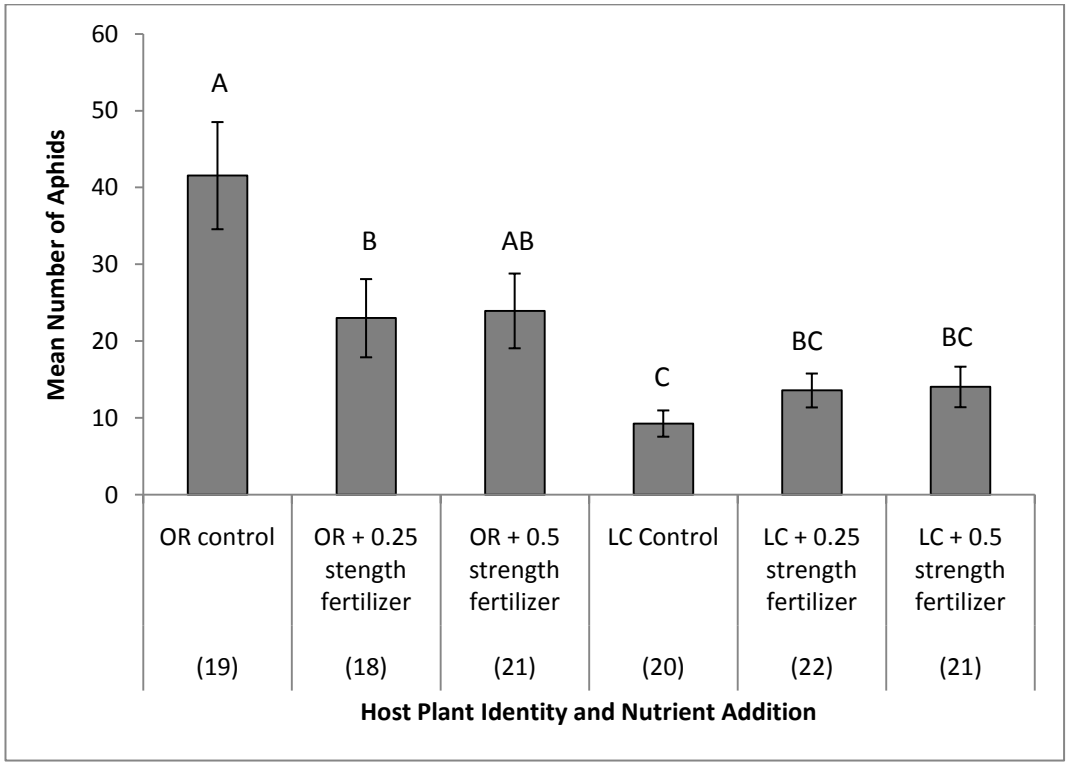

Figure 3. The mean number of Aphids after 14 days that were feeding on *R. minor* plants grown with either *Lotus corniculatus* (LC) or *Ononis repens* (OR), and receiving either no additional fertilizer (control), 0.25 strength Hoagland's solution or 0.5 strength Hoagland's solution (starting density = 20 aphids). Error bars show ± 1 SEM. Replicate number is shown in parenthesis below each treatment. Two-way ANOVA; nutrient addition, $F_{2,115} = 0.15$ $P = 0.861$; host identity, $F_{1,115} = 31.28$ $P < 0.001$; interaction, $F_{2,115} = 6.28$ $P = 0.002$. Letters above bars distinguish significantly different values (Tukey *post-hoc* analysis $P = 0.05$). Data were $\log_{10}$ transformed to meet the assumptions of the test.

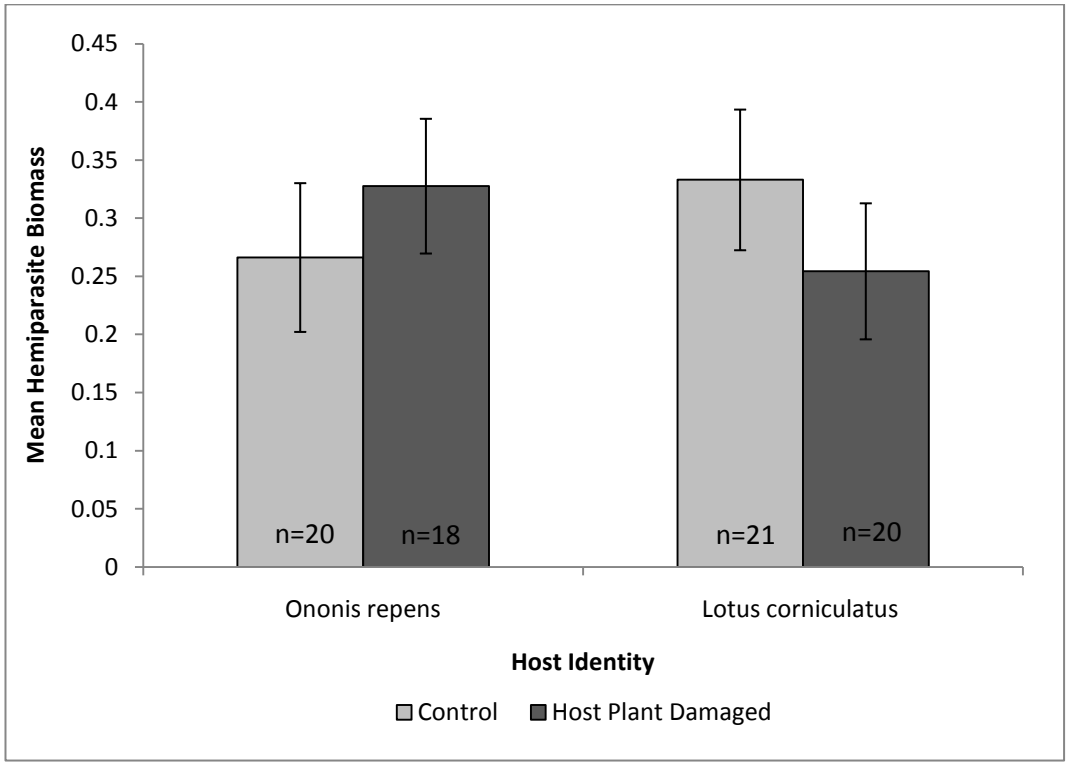

Figure 4. The mean biomass (g) of *Rhinanthus minor* grown with either *Lotus corniculatus* or *Ononis repens* which in turn had received weekly mechanical damage or were left undamaged (control). Error bars show ± 1 SEM.

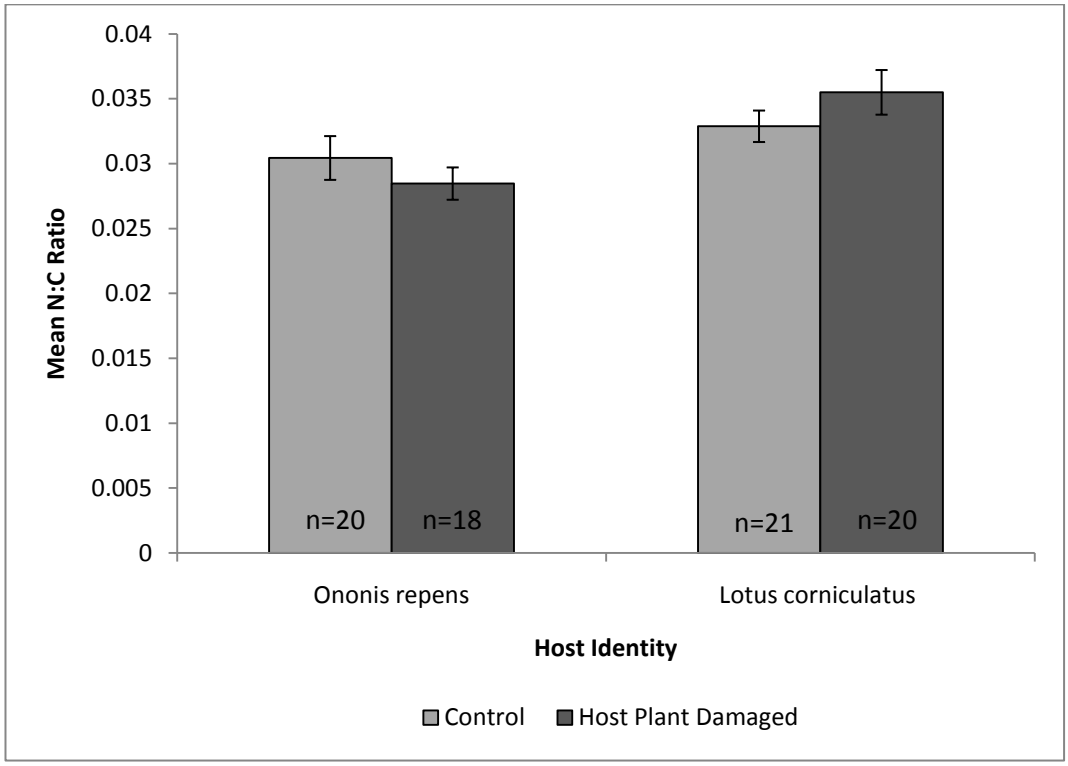

Figure 5. The mean Nitrogen to Carbon ratios of *Rhinanthus minor* grown with either *Lotus corniculatus* or *Ononis repens* which in turn had received weekly mechanical damage or were left undamaged (control). Error bars show ± 1 SEM.

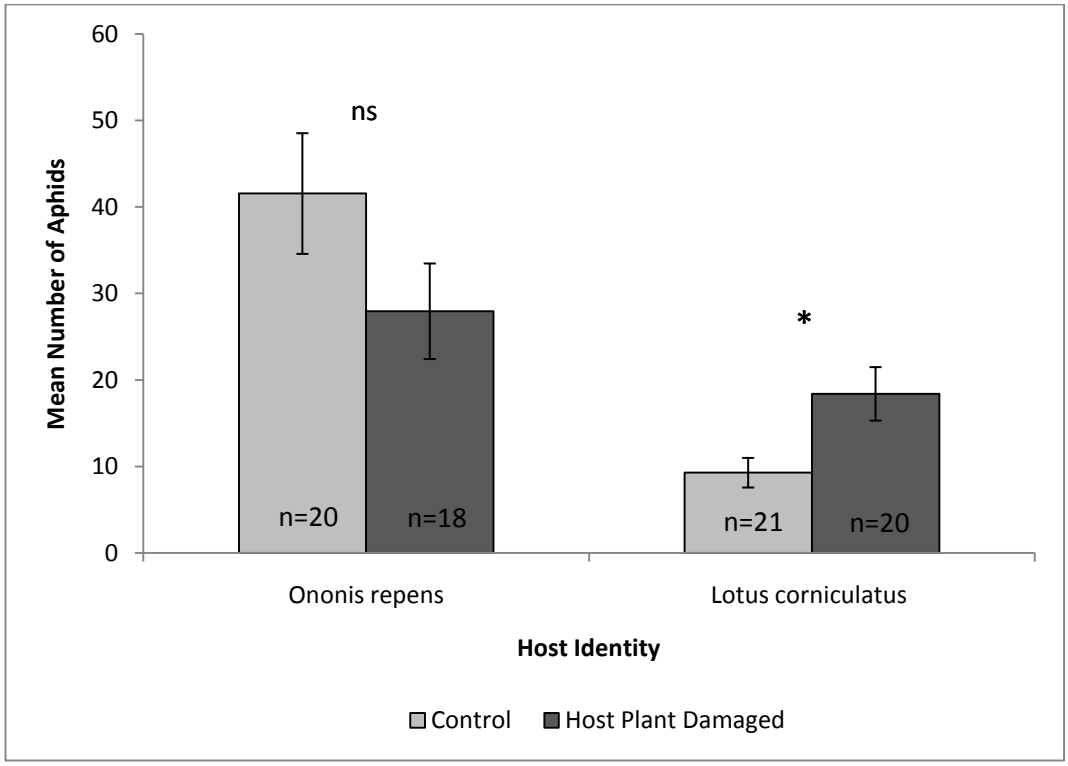

Figure 6. The mean number of Aphids after 14 days that were feeding on *R. minor* plants grown with either *Lotus corniculatus* or *Ononis repens* which in turn had received weekly mechanical damage or were left undamaged (control). Error bars show ± 1 SEM. ns indicates there was no significant difference: t-test; $t_{36} = 1.90$, $P = 0.065$. * indicates a significant difference: t-test; $t_{39} = -2.48$, $P = 0.018$. Data were $\log_{10}$ transformed to meet the assumptions of the test.

Chapter 6. Discussion

6.1 Introduction

The hemiparasite *Rhinanthus minor* is an important component of many grassland ecosytems, having major impacts not only on its host plants (Cameron *et al.*, 2008, Jiang *et al.*, 2003) but on entire plant communities (Gibson and Watkinson, 1989, Joshi *et al.*, 2000, Pywell *et al.*, 2004). These impacts have led several authors to suggest that *R. minor* could be used as a management tool for increasing diversity in species poor grasslands (Davies *et al.*, 1997, Westbury *et al.*, 2006, Bullock and Pywell, 2005). However, to-date, relatively little is known about the interactions between this parasitic plant and its invertebrate herbivores. The primary aim of this thesis was to examine several of the ecological factors that may affect *R minor*, and to investigate how these factors impact on the interactions between the hemiparasite and its invertebrate herbivores. The key findings of the thesis were:

- Increasing *R. minor* density negatively impacts on its performance and survival, and has increasingly dramatic impacts on the surrounding plant community.

- Host identity strongly affects the growth and performance of *R. minor*, and also has major impacts on the growth and performance of its invertebrate herbivores.

- Attachment to multiple hosts is beneficial to the performance of the hemiparasite, and the impacts of certain host species on the invertebrate herbivores of *R. minor* are still apparent when the hemiparasite is also attached to another host plant species.

- The impacts of host plant damage and increased levels of fertilizer in the growing medium can have significant impacts on the invertebrate herbivores of *R. minor* while having negligible impacts on the performance of the hemiparasite itself.

While these key findings, together with the questions laid out in the introduction, have all been discussed in some detail within the individual chapters, the aim of the current chapter is to discuss some broader questions relating to the ecology *R. minor*. This chapter also aims to present some ideas for future work in this area and to demonstrate the importance of this thesis to our understanding of the ecological interactions of *R. minor*, and to our understanding of parasitic plants as a whole.

6.2 What are the challenges of feeding on *Rhinanthus minor* compared to other plants?

Invertebrate herbivores are faced with a broad range of challenges and perhaps most notable amongst these is the poor nutritional quality of plants as a food source (Hartley and Jones, 1997). This is principally because plants are a relatively poor source of nitrogen for invertebrate herbivores (Douglas, 1993, White, 1984, Mattson, 1980), and because they deter herbivores with a multitude of physical (Lucas *et al.*, 2000, Hanley *et al.*, 2007) and chemical defences (Bennett and Wallsgrove, 1994). Hemiparasites present an especially significant challenge to invertebrate herbivores because the vast majority have the ability to attach to wide a range of host plant species (Kuijt, 1969). Because the quality of these host plants for the hemiparasite can be extremely variable (Marvier, 1996, Seel and Press, 1993), they can therefore, have profound impacts on the growth and nutrient content of the hemiparasite, and

hence nutritional quality for its invertebrate herbivores. The experiments presented in both Chapters 3 and 4 clearly demonstrate that this is true for herbivores feeding on the hemiparasite *R. minor*. The results from these experiments show that the identity of the host plant strongly affected the performance of the hemiparasite and the relative amount nitrogen in their tissue, and this was directly related to the performance of the hemiparasites invertebrate herbivores. For example, a correlation between the Nitrogen to Carbon ratio of *R. minor* and the performance of aphids feeding on those hemiparasites was significant for eight of the nine host plant species investigated here. Furthermore, *R. minor* is capable of forming attachments to as many as 50 different host plants from a wide variety of families (Gibson and Watkinson, 1989). Therefore, in terms of its nutrient content and available biomass the hemiparasite *R. minor* represents a highly variable and inconsistent food source for invertebrate herbivores.

Hemiparasites present another significant challenge to invertebrate herbivores because of their ability to acquire defensive secondary metabolites from their host. This phenomenon has been demonstrated in several hemiparasite species, and unsurprisingly can have major negative impacts on the hemiparasite's invertebrate herbivores (Marvier, 1996, Adler, 2002, Lehtonen *et al.*, 2005). The work presented in Chapters 3, 4 and 5 strongly suggest that this also occurs in the interactions between *R. minor* and the host plant *Lotus corniculatus*. In this example, the experiments from all three chapters showed that despite *R. minor* plants attached to *L. corniculatus* having the highest nitrogen content of all the host-hemiparasite associations, the performance of the aphids and snails feeding on the hemiparasites attached to *L. corniculatus* was significantly reduced. Furthermore the impacts of this unidentified metabolite on *R. minor's* invertebrate herbivores are still apparent

even when the hemiparasite is also attached to another plant species. Because *R. minor* has a very broad host range, it seems unlikely that this ability to acquire defensive compounds from its hosts is restricted to this one species. Therefore, in addition to its highly variable nutrient content, *R. minor* may contain a spectrum of secondary defences acquired from a range of hosts.

All plants represent a variable food source for invertebrate herbivores to some extent because a wide range of environmental factors can influence their nutrient content and their defence allocations. These factors include the impacts of increased soil nutrients and light availability (Bryant et al., 1983), drought stress (Masters *et al.*, 1993, White, 1984), and the impacts of damage from other herbivores (Karban and Baldwin, 1997, Kaplan *et al.*, 2008). Hence plants are a variable and complex resource for herbivores (Krischik and Denno, 1983, Denno, 1983), herbivores feeding on *R. minor* face additional variation due to the indirect impact of being attached to different/alternative host plant species. In effect *R. minor* represents an "indirect food source" for invertebrate herbivores because of its close relationship with its host plant's chemistry. For example, the investigations presented in Chapter 5 suggest that some factors which can indirectly affect invertebrate herbivores can feed through *R. minor* and have significant effects on the performance of the hemiparasite's invertebrate herbivores, while having very little effect on the hemiparasite itself. In this example both the addition of nutrients and mechanical damage of the host plant had little or no effect on the performance of the hemiparasite but significantly affected its invertebrate herbivores.

The work presented in this thesis suggest that although *R. minor* may represent a nitrogen-rich food source for invertebrate herbivores, often with a high abundance

within the plant community, its nutritional quality is little more than a lottery depending its host, the presence of additional hosts and factors affecting its host such as nutrient availability and damage. The highly unpredictable nature of the interactions of *R. minor* and its host make it a very challenging proposition for its potential herbivores.

6.3 Why are there so few specialists associated with *Rhinanthus minor*?

The vast majority of insect herbivores (>90%) are thought to be specialists in that they utilise only one, or a few closely related species of plant as a food source (Bernays and Graham, 1988). Both the biological flora (Westbury, 2004) and Phytophagous Insect Database seem to suggest that the hemiparasite *Rhinanthus minor* has very few specialist invertebrate herbivores associated with it (just four species). There would seem therefore, to be a relative paucity of specialist insect herbivores associated with this plant.

This can perhaps be partially explained by the process of plant-herbivore co-evolution, which can lead to herbivores becoming specialised on a certain plant species (Berenbaum, 1983). The process essentially involves the evolution of a specific defence, often a chemical defence, followed by the evolution of a mechanism overcoming this defence. The evolution of specialism provides several important advantages to the insect herbivore, most notably that it has a food source that is relatively free from competition, and that it allows the herbivore to utilise their food source more efficiently (Scriber, 1983). In the case of *R. minor* and other hemiparasites the majority of chemical defences are not produced by the plant itself but are imported from their host plant. As discussed above, the presence, identity and

concentrations of defensive compounds in the hemiparasite are largely unpredictable, meaning that its chemistry is somewhat more than a moving target (Adler and Karban, 1994), and making specialization a considerable challenge. In addition to this highly unpredictable nutrient content of the hemiparasite, as shown in Chapter 3, makes it a risky proposition for a specialist.

While the hemiparasite represents a poor choice nutritionally for a specialist herbivore, the annual life history strategy and population dynamics of *R. minor* can also potentially explain why it has so few specialists. Firstly, as was demonstrated in Chapter 3 and by several other studies (Gibson and Watkinson, 1989, Joshi *et al.*, 2000, Pywell *et al.*, 2004), the hemiparasite *R. minor* can have major impacts on the composition of the vegetation. At very high densities these impacts can be very severe leading to dramatic decrease in the survivorship of the hemiparasite, and presumably a decrease in its nutritional quality, although it should be noted that in chapter two there was no evidence for this. Furthermore, the dramatic changes in the composition of the vegetation caused by *R. minor* are thought to make the resulting habitat less suitable for the hemiparasite. This can lead to populations or *R. minor* moving though the habitat like a wave (Cameron *et al.*, 2009), meaning that it is potentially challenging for any invertebrate to locate. In addition to this, because the viability of *R. minor* seed is limited to single year, the hemiparasite possesses no long-term seed bank (Westbury, 2004). Therefore, any event that leads *R. minor* to being removed before it sets seed, such as heavy grazing or mowing, will ultimately result in its complete loss from the habitat, leaving any specialist herbivores without a food source.

Overall, it would appear that the paucity of specialist herbivores associated with this hemiparasite is not surprising. Relatively few studies have characterised the invertebrates feeding on *R. minor*, so a more thorough investigation into the invertebrate herbivores feeding on this plant is required before any firm conclusions can be drawn. It is worth noting that during the preliminary work of this thesis, an investigation of the hemiparasites at Castle Hill National Nature Reserve (Grid ref: TQ 375 065) revealed no specialist herbivores feeding on *R. minor*.

6.4 What are the responses of different feeding guilds to host plant identity?

Numerous studies have demonstrated that factors affecting the palatability or nutritional content of plants for invertebrate herbivores differentially affect invertebrates from separate feeding guilds (e.g. Massey *et al.*, 2006, Macel *et al.*, 2005, Bass, 2004). Interestingly however, the experiments presented in Chapter 3 showed that the impacts of host plant identity on their invertebrate herbivores appeared to be very similar for herbivores from three separate feeding guilds (with the notable exception of invertebrates feeding on hemiparasites attached to the host plant *Lotus corniculatus*). This result demonstrated that for the majority of host plant-hemiparasite combinations, the most important factor for all three feeding guilds of invertebrate herbivores feeding on *R. minor* was growth and/or nitrogen content of the hemiparasite, which in turn depended on the host's identity. When the hemiparasite was attached to *L. corniculatus*, however, both the phloem feeding and leaf chewing invertebrates showed a significantly reduced performance, while the xylem feeder was unaffected. The underlying reasons for these differences are hard to predict without knowing the identity of any possible defensive compounds passing

from *L. corniculatus* to *R. minor*, or indeed whether there was such a compound involved. Assuming a secondary metabolite was responsible, three possible reasons for these different responses between feeding guilds present themselves. Firstly, the xylem sap may contain concentrations of the defensive metabolite which are low enough not to affect the xylem feeder. Secondly, the metabolite may be in an inactive form while present in the xylem and must be converted elsewhere before it can negatively impacts on the invertebrate herbivores. Finally, the xylem feeder in question may not be affected by this metabolite. Indeed this xylem feeder, *Philaenus spumarius,* is known to feed on *L. corniculatus* (Jones and Turkington, 1986) suggesting that this could be the case.

Factors other than host identity shown in this thesis to affect both the hemiparasite and certain invertebrate herbivores, such as nutrient availability, were not extended to include a range of feeding guilds. To explore fully the impacts of multiple hosts, host damage or increased nutrient supply on the invertebrate herbivores of *R. minor,* and to what extent these vary between feeding guild additional experimental investigations will be required.

6.5 What are the potential impacts of *Rhinanthus minor* on invertebrate herbivore communities and by what mechanisms do they operate?

The hemiparasite *R. minor* has been shown in this thesis (Chapters 2 and 3) and by other authors (Jiang *et al.*, 2003, Cameron *et al.*, 2008) to reduce its host performance. At a community level the reduction of host plant biomass, or indeed the reduction in the biomass of the whole plant community, as a result of the presence of a hemiparasite (Ameloot *et al.*, 2005, Pywell *et al.*, 2004), together with

changes on the composition of the plant species present (Pywell *et al.*, 2004, Joshi *et al.*, 2000, Davies *et al.*, 1997) are likely to have profound effects on the invertebrate community. These effects may be caused by a reduction in the quantity or quality of the available host plants, or because of competition between the hemiparasite and the herbivore for shared hosts (Bass, 2004), or they may be due to the presence of a wider a range of plants, thereby increasing the number of specialist herbivores.

The impact of the hemiparasite on its host may, however, have some other important impacts which may change the feeding preferences of invertebrate herbivores. For example, work by Puustinen and Mutikainen (2001) showed that the attachment of a hemiparasite decreased the levels of cyanogenic glycosides in the host plant *Trifolium repens* making this plant more palatable for invertebrate herbivores. Such changes in the chemistry of the host plants may well lead to an increase in herbivore abundance in the area directly around the infected plants.

Such changes may also have implications for nutrient cycling as well as directly affecting invertebrate herbivores. For example, cyanogenic glycosides are not only important defensive secondary metabolites but also important nitrogen storage molecules (Poulton, 1990). Therefore, decreases in the levels of cyanogenic glycosides in the host plant due to the attachment of a hemiparasite (Puustinen and Mutikainen, 2001) may lead to nitrogen being removed by the hemiparasite that would otherwise have been stored in the host. Indeed, parasitic plants have been shown by several authors to have major impacts on nutrient cycling (Quested, 2008, Bardgett *et al.*, 2006, Ameloot *et al.*, 2008), and these impacts are also likely to affect not only the plant community but also its invertebrate herbivores (Mattson, 1980, Bryant *et al.*, 1983). Therefore, the mechanisms underlying the impacts of

hemiparasites on nutrient cycling and its potential effects on the invertebrate herbivore community is an area that warrants further attention.

6.6 Is a metabolomic approach required in order to fully understand to the interactions between *Rhinanthus minor*, its hosts and its invertebrate herbivores?

While the experimental work presented in this thesis has provided answers to some important questions on the ecology of this hemiparasite, several other aspects of *R minor's* ecological interactions remain in question. Several of these points have been discussed in the previous sections of this chapter. The identity of the secondary metabolite responsible for the decreases in performance of aphids and snails feeding on the hemiparasite attached to the host plant *L. corniculatus* (shown in Chapters 3, 4 and 5) remains unknown. While there are numerous candidates, the presence and/or identity of any secondary metabolite(s) in this system remain speculative. It seems likely therefore, that a metabolomic profiling approach will be required in order to clarify the underlying mechanisms underpinning this interaction. Indeed, such an approach could well be applied to both host and hemiparasite. The negative indirect impacts of a hemiparasite on the performance of its host plants (Cameron *et al.*, 2008, Matthies, 1995, Seel and Press, 1996, Tennakoon and Pate, 1996) and the invertebrate herbivores feeding on those plants (Bass, 2004) may well be caused predominantly by water and nutrient loss. There are, however, indications that host plants undergo more fundamental changes in their chemical composition when under attack by parasitic plants (Puustinen and Mutikainen, 2001, Bass, 2004, Seel and Jeschke, 1999). Therefore a careful inspection of the metabolome of *R. minor* plants attached to various hosts, and the impacts of the *R. minor* on their host's metabolome

would be an important advance in our understanding of the interactions between hemiparasites and their hosts.

6.7 Conclusions

The data presented in this thesis have demonstrated that the hemiparasite *Rhinanthus minor* can have important impacts on plant communities and is a highly variable food source for invertebrate herbivores. Most previous work examining the indirect impacts of host plants on the herbivores of hemiparasites has focussed on interactions known to involve secondary defences (e.g. Adler, 2000, Marvier, 1998). The results presented here have demonstrated that while some host can influence their hemiparasite's invertebrate herbivores via secondary defences, other factors such as the performance of the *R. minor*, or its carbon:nitrongen ratio can also have important impacts on invertebrate herbivore performance. Furthermore, while host identity appears to be the most significant factor affecting these interactions, the other factors examined here can also have important impacts. Overall the work presented in this thesis provides a clearer understanding of the ecological interactions between this hemiparasite, its host and its invertebrate herbivores. Furthermore, this work adds a considerable body of information not only to our understanding of *Rhinanthus minor* but also to our understanding of a wide range of ecological interactions involving parasitic plants.

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